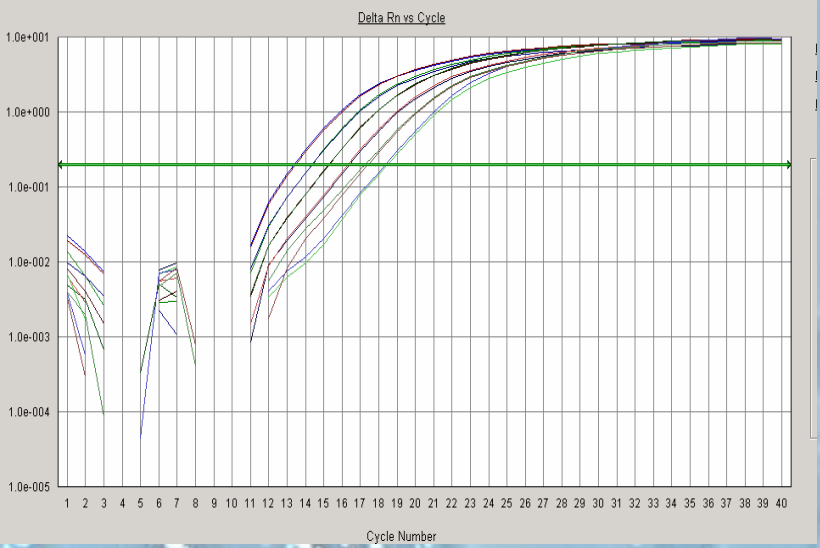
"Expression of pro-inflammatory cytokines in Atlantic salmon (*Salmo salar)* after intraperitoneal injection of PLGA[Poly(D-L-lactide-co-glycolic) acid] particles"

By
Stine Mari Myren



Master in Aquamedicine
Department of Marine Biotechnology
Norwegian College of Fishery Science
University of Tromsø
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Table of Contents

1. Introduction 1

1.1. Innate immunity in fish 1
1.1.1. The acute phase response 5
1.2.1. Pro-inflammatory cytokines 5
1.2. Vaccines in intensive fish farming 8
1.2.1. Poly (D-L-lactide-co- glycolic) acid (PLGA) as a vaccine carrier. 9
1.3. Objectives 11
2. Materials and methods 13

2.1. Materials 13
2.1.1. Animals 13
2.1.2. Lipopolysaccharide (LPS) 13
2.2. Methods 13
2.2.1. Making of PLGA particles 13
2.2.2. Characterization of PLGA particles 14
2.2.3. Distribution of PLGA particles in tissue after i.v. injection. 15
2.2.4. Gene expression profiling after ip injection 17
3. Results 25

3.1. PLGA characterization 25
3.1.1. Surface characterization 25
3.1.2. Size distribution of PLGA particles 25
3.2. PLGA particles associated with ip cells after ip injection 26
3.3. Tissue distribution of PLGA particles after i.v. injection 27
3.4. Cytokine expression after ip injection 27
3.4.1. Expression of IL-1β 27
3.4.2. Expression of IL-6 30
3.4.3. Expression of IL-8 33
3.4.4. Expression of TNF-α1 36
3.4.5. Comparison of TNF-α1, IL-1β1, IL-6 and IL-8 expression 39
4. Discussion 47

4.1. PLGA in association with ip-cells 47
4.2. Cytokine m-RNA transcripts 48
4.2.1. Tools for real time-PCR 48
4.2.2. mRNA transcript levels post ip injection 49
4.2.3. Experiment set-up and materials 50
4.3. Distribution of PLGA particles 52
4.4. Future thoughts 52
5. Conclusion 54

6. Appendix 55

Appendix 1: Production data 55
Appendix 2: Chemicals and reagent 56
Appendix 3: Solutions 57
Appendix 4: Equipment 59
7. References 63

Summary

Vaccines in aquaculture are heading into the 21st century facing old challenges with new possibilities. Fish die each year as a result of inefficient vaccination against intracellular pathogens e.g. infectious pancreatic necrosis virus (IPNV). A successful prophylactic strategy to combat viral diseases like IPN in fish farming depend both on innate immune responses, like cytokines and natural killer cells, and on specific responses, like antibodies and cytotoxic T cells. In new vaccine strategies for fish, knowledge of how to effective stimulate innate immune responses is essential.

In this study we have investigated the expression of pro-inflammatory cytokines after injection of empty fluorescent Poly(D-L-lactide-co-glycolic) acid particles (PLGA) to see what affect PLGA particles have on the immune response per se.

Four groups of Atlantic salmon of ~80 g were intraperitoneally injected with respectively NaCl (0.9%), LPS (1mg/kg), PLGA (10^8 particles/fish) and a mixture of PLGA (10^8 particles/fish)/LPS (1mg/kg). Tissue and cell samples were collected at day 2, 4, 7, 14 and 30 post-injection. Cell samples were taken from head kidney and peritoneum, and tissue samples from liver and spleen.

The expression of the pro-inflammatory cytokines, IL-1b, IL-6, IL-8 and TNF-α1 in peritoneum, spleen, liver and head kidney macrophages was measured using Real time Reverse transcriptase Polymerase chain reaction (Real-time RT-PCR).

In head kidney macrophages and peritoneum the expression levels in the 3 experimental groups, injected with PLGA, LPS and a PLGA/LPS mix were low throughout the whole sampling period. Expression of IL-6 in liver was too low to be detected in all 3 experimental groups and also in the saline injected fish. The results from spleen and liver of fish injected with PLGA/LPS and LPS showed elevated levels of especially TNF-α1, IL-6 and IL-8 at early stages (2-4 days), and overall elevated mRNA transcript levels were detected at early stages.

The particles were labelled with 6-coumarin, for a visual study of intraperitoneal (ip)-cell samples. Fluorescent PLGA particles were microscopic visualized in connection to ip-cells up to 14 days post-injection. An attempt to evaluate distribution patterns of PLGA particles in different tissues did not succeed.

1. Introduction

1.1. Innate immunity in fish

Immunology is "the study of the bodies´ physiological defense against what the body considers as non-self or alien/foreign" [1]. One refers to this physiological defense as the immune system, because of the many different cells that interact to give responses to invading micro-organisms. Leukocytes are the main cells of the immune system and are localized to certain organs and tissues [1]. Bone fish have lymphoid organs as head kidney, thymus, spleen and some lymphoid tissue, but do not possess lymph nodes and bone marrow as mammals do [43, 75]. The head kidney is considered to be the most important lymphoid organ as it is haematopoietic [22, 43]. Circulation in between the lymphoid organs occurs in the blood and lymph-veins.

The immune system of higher vertebrates can be divided in two sections; the innate (IIS) and the adaptive (AIS) immune system [1, 20, 67]. These to sections often cross each other in functions and work together to give an effective immune response and innate immunity is crtitical for activating adaptive immunity. From a phylogenetical point of view, fish are one of the first classes of vertebrates that evolved to possess an adaptive part of the immune system [74], with teleosts being the most developed class of fish [67, 75].

The IIS in fish consist of physical barriers like mucus, scales, mucosal surfaces (epidermis, gills, intestine) that provide an obstruction to invaders. In addition the mucus of fish may contain immune factors like lectins, pentraxins, lysozyme, complement proteins, antibacterial peptides and immunoglobulin M (IgM) [53]. Secondly, the IIS provides different cells to handle invaders (Table 1). Monocytes/macrophages and granulocytes (neutrophils) are the key cells of the innate defence system in fish [2, 23, 64], and as shown in mammals, are recruited to the site of infection by messenger proteins (e.g. chemokines). Macrophages and granulocytes are also located in other tissues than the kidney. The resting population of leucocytes in the peritoneum i.e. of fish has been shown to consist of macrophages (40%), leukocyte-like cells (55%) and neutrophils (2%) [23]. In response to an intraperitoneal (ip) injection of bacteria, a high influx of neutrophils and are seen after 24-48 h the neutrophils has been shown outnumber the macrophages in the peritoneal cavity [2]. Neutrophils are short-lived phagocytising and active secreting cells, while macrophages are the more long-lived, that may stay at the site of infection. Also the spleen is an important site for maturation

of leucocytes, and antigens are considered to be taken up by the endothelial reticular cells in the ellipsoids of the tissue [80].

After entering the extravascular site the monocytes may differentiate into macrophages [1]. Monocytes and macrophages are two stages of the same cell lineage of phagocytic cells, called mononuclear phagocyte system (MPS) [1]. Macrophages will recognize general pathogen characteristics normally not found on the cell surface of any eukaryotic organisms, that are highly conserved in a wide range of micro-organisms, e.g. bacterial and fungal moieties such as lipopolysaccharides (LPS) and ß-glucan [53]. Collectively these patterns are called Pathogen Associated Molecular Patterns (PAMPs) and macrophages "see" them by surface receptors [1, 3, 42], also known as pattern recognizing receptors (PRRs). Macrophages can be activated both through this recognition of PAMPs and also by interferon-γ and cytokines produced by themselves or other cells [1, 54]. Besides antigen presentation, the action of macrophages are to modulate other cells action by secreting products like enzymes, antimicrobial peptides, oxygen- and arachidonic acid metabolites and

Table 1: Examples of components of the innate immune system of teleosts [53]. APP: acute phase protein. TLR=Toll like receptor (a main receptor among PRRs, others are, i.e.mannosereceptors)

Innate immune components in teleosts	**Mode of action**
Physical components	
Fish scales	Physical obstacle
Mucous surfaces	Viscous glycoproteins gives a physical obstacle, and contains antiseptic enzymes
Non-cellular (humoral)	
Transferrins	Iron-binding APP, making an iron-free environment, depleting the iron-source from pathogens. Growth inhibitor.
Lectins	Carbohydrate-binding, recognizes carbohydrate moieties on bacteria
C-reactive protein (lectin)	APP, levels increases in inflammatory responses, binds to phosphorylcholins on microbes and assists in complement binding
Lytic enzymes	These enzymes are innate opsonins, changing the surface charge of microbes and facilitating phagocytosis
Anti-bacterial peptides, proteins	Effect on bacteria, either bacteriostatic or bacteriolytic
Interferons/MX-proteins	Inhibiting viral replication
Enzyme inhibitors	Blocking and inactivating/decreases enzyme activity
Mucus	See above, and page 1
Complement	Biochemical cascade that may lead to removal of pathogens
Cellular	
Natural killer cells (NK)	"Kill cells" missing MHC class I molecules on cell surface
Phagocytic cells with TLR and "burst" activity (E,g. macrophages and neutrophils)	PRR harbouring and exhibit many different responses

cytokines, and to actively phagocytose microbes [1, 80].

An important family of receptors which are found to recognize different entities in the first encounter of microbes, are the Toll-like receptors (TLR) [3, 42]. The TLR family has receptors for nucleic acid, proteins, lipids and polysaccharides. The recognition often initiates endocytosis of the alien agents and results in intracellular signalling with production of several cytokines.

There are found 10 different TLR in mammals, in fish there has been identified more [37]. Some TLRs work with different ligand specificities and expression patterns and knowledge of how different TLR`s function are important. In mammals the TLR 4 is the main receptor for LPS [34]. LPS is an important constituent in the outer layer of Gram-negative bacteria, and it consists of 3 parts; an O-specific polysaccharide, a core polyoligosaccharide and a lipid A. The lipid A-layer is responsible for activation of innate immune responses in mammals, also referred to as the endotoxin in LPS [52]. In mammals, the binding of LPS to TLR is acchieved through a complex of factors (Fig. 1), and this triggers an intracellular cascade dependent on MyD88, which leads to removal of the inhibitor of NF-kB and release of p50 and p65 subunits which again enters the nucleus and drive the expression of pro-inflammatory genes [28]. LPS binding also leads to antiviral gene expression through an alternative pathway independent of MyD88 [37]. Comparative studies have shown that fish and lower vertebrates are resistant to the toxic effect of LPS that in mammals can cause a septic shock [7].

In vitro studies in fish show that there is a need for extremely high LPS concentration to induce immune responses in comparison to mammals [51], and this could be due to differences in their recognition of LPS [36]. Some studies suggests that fish lack some components in the TLR 4 pathway or use a different receptor pathway, and this leads to an attenuated LPS sensitivity [37]. In addition there is no trigging of antiviral genes after LPS challenge in fish, which can suggest another signalling pathway being used [37]. There are also questions on how well TLR4 genes are conserved in across lower vertebrates, and although zebrafish, *Danio rerio*, do show homologues, this is not shown in e.g. pufferfish, *Fugu* and *Tetradon*, [37].

Lastly, there is the humoral (non-cellular) response in fish (Table 1), and according to Magnadottir (2006), this part is classified according to the components impact on PRR or function specifity.

In mammals, the innate immunity is fundamentally important for an effective adaptive immune response to occur [24], and this is also the case in fish [20]. It could also be that teleost need to rely more on the innate immunity when opposing pathogens [5, 6, 23, 38] since the humoral immunity of fish is deficient of e.g. IgG and IgE. In fish and other ectothermic vertebrates the adaptive part of the immune system is temperature dependent, and thereby limiting, since a high antibody response may be necessary for a protective outcome [48]. Temperature and time to develop specific immunity are inversely related, the lower temperatures of the fish the longer time it will take for the adaptive immunity to develop [5, 23]. Antibody production after vaccination under even optimal temperatures for salmon (12-14^{O}C) is known to start after 4-6 weeks compared to fish living in warm-waters of 22^{O}C, where antibodies are detected after one week post-immunization [23]. It is a disputed issue whether the innate response in fish is delayed due to a lowered temperature. Some findings support that the innate immunity in fish is affected by temperature, and that fish react fast to PAMPs [4, 12, 57]. Other findings in rainbow trout suggest otherwise [66].

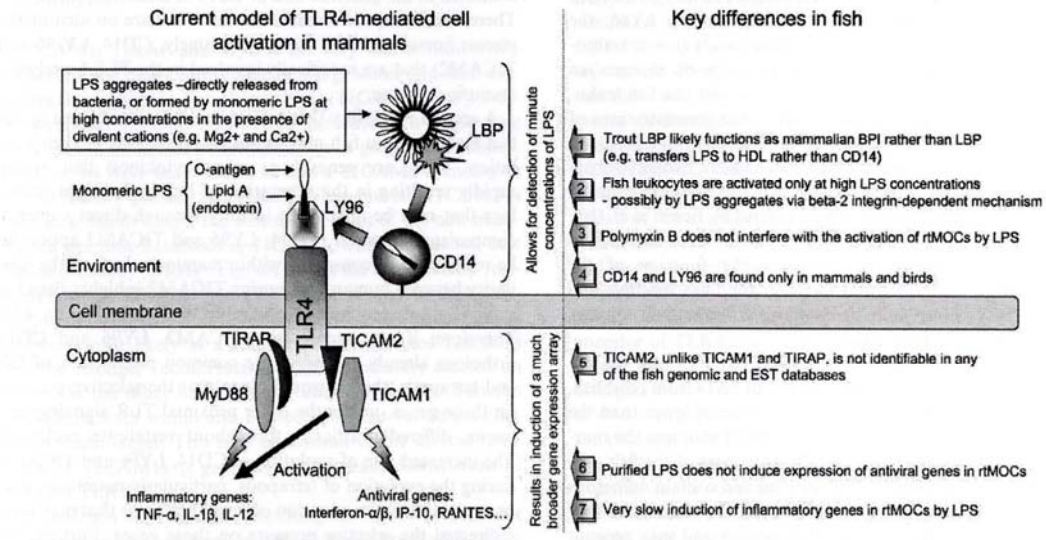

Figure 1: Toll-like receptor 4 – complex and the MyD88 dependent pathway. LPS` in the blood binds either directly or by LPS-binding proteins (LBP) in the blood to a CD 14 (membrane-linked protein) and TLR4. Together with MD2 a signal cascade through Myeloid differentiation factor 2 (MyD88) is initiated and eventually leads to transcription of pro-inflammatory cytokines like TNF-α, IL-1,IL-8 and IL-6 through the NF-κB pathway [37].

1.1.1. The acute phase response

In response to tissue damage, stress or microbiological intruders an inflammatory response is initiated. This first response is referred to as the acute phase response (APR) [6, 26]. The APR response is recognized by an increase in plasma concentration of different acute-phase proteins. The different APP products have various functions, such as transferrins and metallothenins that are responsible for depleting the pathogens of nourishment sources, while some are taking part in maintaining homeostasis and regulations, and others are transport proteins [5, 77] The acute phase normally starts within hours and subsides within 24-48 h [11]. After this the organism will return to normal. The acute phase response may persist and turn into to a chronic inflammation. In fish this acute phase response may be delayed due to environmental temperature as mentioned and it can take more than two days for it to subside [88].

The cardinal signs of inflammation are redness (rubor), heat (calor), swelling (tumor), pain (dolor) and the tissues involved show a lack in functionality (function laesa). Inflammation can be divided in two sections [1];

1. The exudative components that have an effect on fluid flow. Blood veins upstream of the inflammatory localisation will increase, sending more blood which will cause heat and redness. The blood veins down-stream will decrease to minimize drainage. At the same time there will be an increase in arterial wall permeability that allows more blood plasma to flow to the site of infection.
2. The cellular part includes leukocytes that migrate to the site of inflammation, and deals with present threats. In response to production of i.e. the pro-inflammatory cytokines IL-1β and TNFα1, the endothelial cells at the site of infection express on their surface enhanced levels of adhesion molecules, selectins and integrins. Each of these molecules enhances specific migration of leucocytes to tissue [1, 85].

1.2.1. Pro-inflammatory cytokines

Cytokines are a group of messenger polypeptides, produced by a variety of cells. Cytokines are important for the immune-cells to co-work, and are considered to have an impact on modulating and directing the immune cells in different ways and for being the chief stimulators of the production of acute phase proteins [26].

Most cytokines have multiple sources, multiple targets and multiple functions (pleiotrophy) [26]. Cytokines are usually products from a TLR mediated signalling and the classical transcription pathway leading to activation of nuclear factor kappa B (NF-kB) [13, 33] This pathway plays a central role in cardiovascular growth, stress response, and inflammation by controlling gene network expression. Cytokines work primarily in a paracrine (short range) and autocrine (on itself) manner through receptors, but they are also shown to work in an endocrine like way [1, 41, 85].

Cytokines responsible for inflammatory responses are often divided in 2 groups [25], those involved in acute inflammation, and those involved in a more chronic inflammation. TNF-α, IL-1β and IL-6 are cytokines most relevant in initializing the APR, but they also play a role in a more chronic inflammation and also in activating the AIS [6, 20]. Primarily pro-inflammatory cytokines are produced by monocytes and macrophages and targets are often different in an inflammatory response [85].

Following inflammation, the cytokine cascade begins with production of TNF-α from macrophages, NK cells or mast cells. TNF-α is usually the first signal of inflammation in fish as it is in mammals [79]. TNF-α have been characterized so far in rainbow trou, *Oncorhynchus Mykiss*, and Japanese flounder, *Paralychtys olivaceus* [32, 45]. In fish this cytokine is known to give a mitogen-like response of head kidney cells and induce activation of fish macrophages with macrophage activation factor (MAF) as a resulting product. TNF-α can be detected in gills, kidney and blood leucocytes of unstimulated fish [45]. Its effect as a pro-inflammatory conductor often overlaps and synergize with the effects of IL-1β, which is one of the key mediators of the body's response to a microbial invasion. IL-1β has been characterized in a large number of bony fish, including rainbow trout [15, 78]. IL-1β may induce a general increase in a wide variety of other factors associated with inflammation and in particular other cytokines [10]. Expression of IL-1ß has shown to be negatively influenced by low temperature and stress [90].

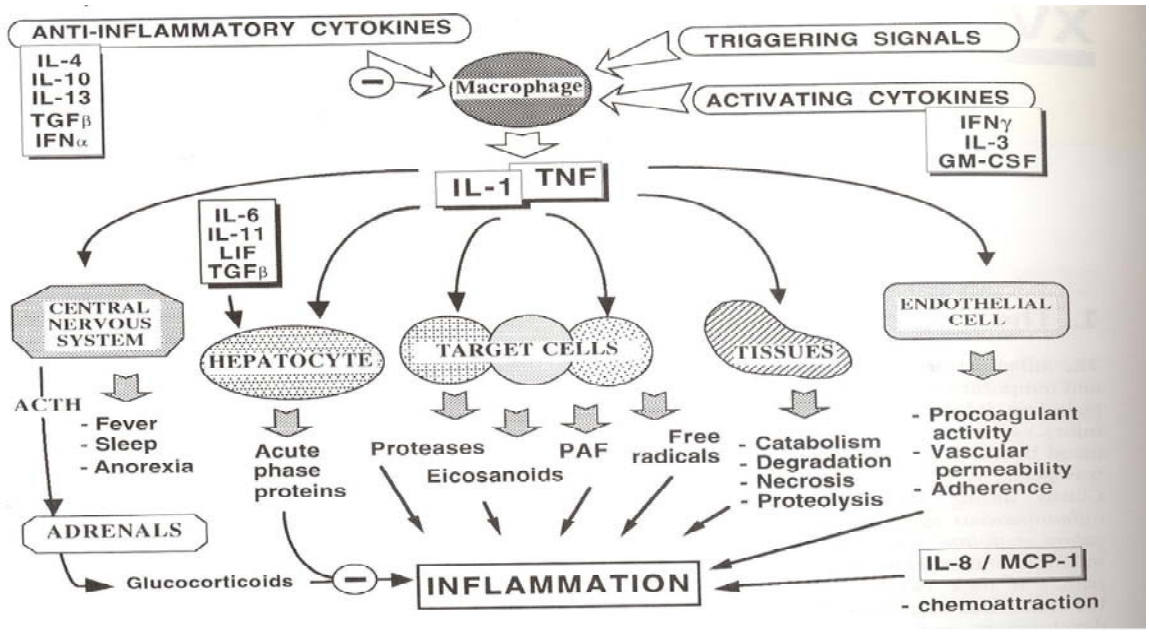

Figure 2: Production of IL-1, IL-6, IL-8 and TNF in the inflammatory response. Triggering signals as stress, cuts, pathogens cause macrophages to produce IL-1 and TNF, and cause a variety of actions due to this cytokine expression. IL-6 is the main conductor for acute phase protein expression and work on the hepatic "side" for this production, whereas and IL-1 TNF also affects the acute phase response. IL-8 as a chemokine will work to attract more neutrophils to the site of infection [85].

Together IL-1β and TNF-α1 induce migration of neutrophils and macrophages by inducing itself, other resident macrophages and neutrophils to produce chemokines like IL-8 at the site of infection [85].

IL-8 is a chemokine or chemoattracting protein. It is the main attributor to activate neutrophils and make sure that they migrate to the site of infection, and also for the activation of degranulation [71]. IL-8 is produced mainly by macrophages and the production is stimulated by cytokines, as mentioned above,(IL-1β and TNF-α1), bacterial products as LPS, viral products (dsRNA) and certain plant products [85]. IL-8 is a very stabile chemokine. It can endure heating up to 100^{O}C, and pH between 2.4 and 9.0 without any significant decrease in its bioactivity [85]. The gene for IL-8 has been sequenced fish, i.e. in Japanese flounder, rainbow trout and channel catfish, *Ictalurus punctatus* [15].

IL-6 is also one of the first cytokines to be produced, and is known to be the main stimulator for the production of acute phase proteins during acute phase response in human, and as an attributor to the migration of neutrophils to the infection site [1, 26, 85]. In addition IL-6 may play a part in Ig-production, and monocyte and lymphocyte differentiation [35, 62]. The cytokine is produced by a diverse group of cells including macrophages, endothelial cells and

T-lymphocytes, neurons and fibroblasts [1, 35]. The gene for IL-6 was first characterized in Japanese pufferfish, *F. rubripes,* in 2005 [9], and its function in fish compared to mammals is not clear yet.

1.2. Vaccines in intensive fish farming

Vaccines consist of non-toxic antigens that are injected, ingested or inhaled to induce a specific defence response, without actually going through the disease process. Either by using components of the pathogen (antigens), attenuated or inactivated forms, one wish to stimulate the protective adaptive immune responses [1, 8].

During the last decades, there has been a continuous growth in the aquaculture industry all over the world [60, 82]. Any intensive bioproduction, whether on land or at sea, will likely suffer from disease problems [27]. Prophylactic treatments and good management practises can usually prevent or reduce the susceptibility to diseases, but not entirely. During the 1980`s the salmon industry experienced great losses due to bacterial diseases, mostly caused by *Vibrio sp*, and the use of antibiotics increased dramatically [49]. The introduction of well-working vaccines in aquaculture has been the main reason for the decrease in antibiotic use [27].

It is shown that attenuated pathogens in most circumstances lack the ability to give a cellular adaptive response that is important to eradicate e.g. virus and other intracellular pathogens, and there is an obvious need for development of such protection [8, 81, 86]. Oil-adjuvanted vaccines, which is the most commonly used vaccine formulation in Norwegian aquaculture [27], have shown sub-optimal efficacy against virus infections (e.g. infectious pancreas necrosis virus (IPNV)) and against some intracellular bacteria like *Piscirickettsia salmonis*. The challenge for vaccine developers, in general, has been to produce vaccines that activate this cellular (Th1) arm of the immune system, that handles intracellular pathogens [27, 63, 81]. In addition, vaccines that increase cytotoxic CD8+ T cell (Tc) responses are also highly wanted. Activation, proliferation and differentiation of naïve T-cells is dependent on presentation of peptide fragments and second signals (cytokines) from APC. APCs are phagocytic cells with the ability to engulf and process these peptide fragments of the antigen, and activating the naive T-cells by externalizing the peptide fragments on a major-histocompatibility complex (MHC). Examples of APCs are macrophages and dendrittic cells.

1.2.1. Poly (D-L-lactide-co- glycolic) acid (PLGA) as a vaccine carrier.

When new vaccine formulations are taken into consideration, there are many aspects to consider; effectiveness in getting the correct immune response is one, while economic aspects and toxicity are others. A highly promising technology is based on polymeric nanoparticles. A particle delivery system consists of a carrier that permits a sustained or pulsed release of encapsulated antigens and adjuvants [50]. Nanoparticles is such a delivery vehicle (To be defined as nano-technology, the size of particles must be <100 nm) with a therapeutic agent of interest encapsulated or adsorbed/conjugated to their surface are of high interest now-a-days. This new nanotechnology devices gives an opportunity to deliver small molecular weight drugs, as well as macromolecules such as proteins, peptides or genes to tissue of interest [58]. By adsorbing different ligands and antigens to the nanoparticle and knowledge of how the adaptive immune response works together with the innate, one can adjust the concept to a desired immune response. PLGA are prepared from lactide and glycolide, which are cyclic esters of lactic acid and glycolic acids [31]. PLGA is synthesized by means of random ring opening co-polymerization of two different monomers, the cyclic dimer (1, 4-dioxane-2,5-diones) of glycolic acid and lactic acid (Fig.3).

In general, the polyester can be made highly crystalline (e.g. poly(L-lactic acid)), or completely amorphous (e.g. poly(D,L-lactic-co-glycolic) acid), and made in almost any micro- and nanosizes, with a capability of encapsulating almost any molecule [39].

PLGA is biodegradable and biocompatible [31], and is approved by the US food and drug administration (FDA) [44], and has shown little or no systemic toxicity [14]. PLGA has been used for ages in medical and pharmaceutical fields, as sutures, bone fixatives, artificial skins and cartilages, dental materials, materials for bone regeneration, drug delivery and others. Today PLGA particles is used in at least 12 different marketed products, and are capable of releasing peptides and proteins slowly and continuously from 1 to 4 months [39].

PLGA particles have been shown to be taken up *in vivo* by the main APCs in mammals, dendrittic cells [65, 89]. and using PLGA nanoparticles as antigen delivery vehicles have shown to enhance antigen-presentation efficiency by 10-100 fold [84], and also increasing

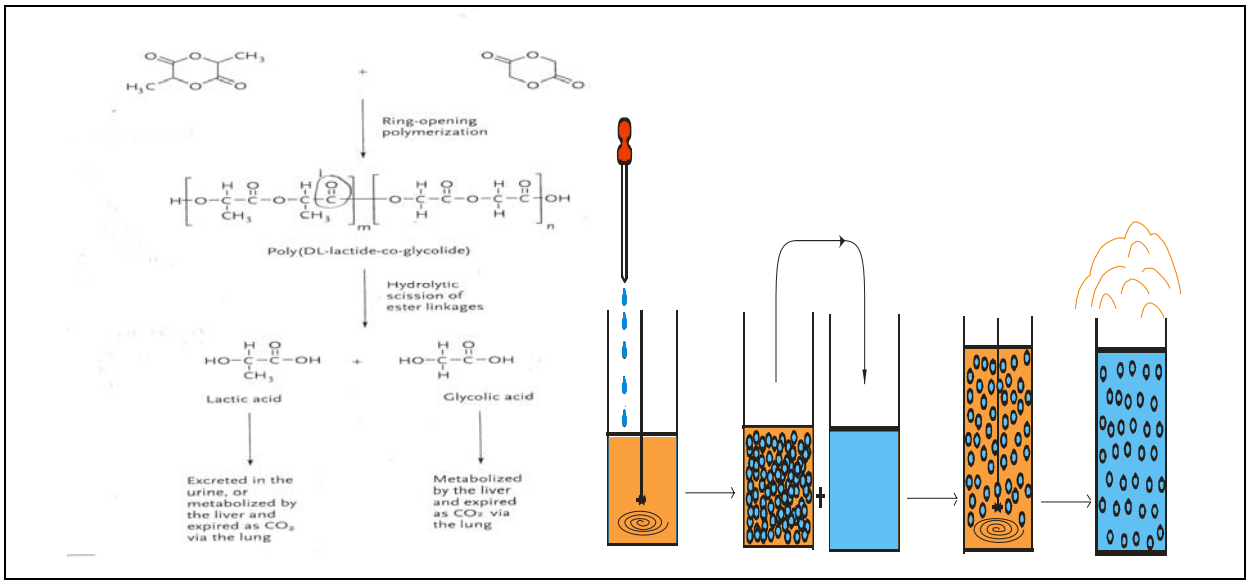

Figure 3: Left: The synthesis of PLGA. PLGA is synthesized from two monomers; glycolic acid (top left) and lactic acid (top right) [31]. Right: Water-in-oil-in-water (W/O/W) emulsification solvent evaporation method is the most used method for making PLGA nano particles. First a water phase (PVA) and antigen is homogenised/sonicated in a polymer solution (PLGA + chloroform), before a second emulsification is included in a second water phase (PVA) and the solution is left stirring for the organic solvent to evaporate.

cytotoxic T-cell activity.

The way of uptake of PLGA nanoparticles has been investigated in mammalian smooth vascular cells and endothelial cells, and the result indicated that the particles are internalized efficiently through an endocytic process and the uptake is concentration- and time-dependent [17, 68]. The process seems to be saturable. The exact way of endocytosis is not determined and it would depend on particle size, ligands adsorbed, and the level of antigen exposed on the surfaces. Adjusting such features may result in an altered organ- and tissue distribution. Studies in mammalian DC also show increased maturation after stimulation of PLGA microparticles alone [89].

A nanosize also gives another merit, it has been shown that 100 nm particles are taken up more easily compared to larger sized particles [19], and can penetrate sub mucosal layers, where larger particles can not [18].

By using PLGA particles or other polymers there is a potential for reducing both the number of administration and the amount of vaccine antigens required to induce protection. A problem in aquaculture in addition to the problems with a sub-optimal Th1 response activation, is side-effects (peritoneal melanization process, internal adhesions and granulomous inflammation), caused by oil-adjuvanted vaccines [61]. Introduction of vaccine

carriers such as PLGA particles may be a mean to decrease such side-effects (not caused by fault-injections).

PLGA particles as a vaccine carrier in fish have only been investigated once [47], then in an oral vaccination try-out for rainbow trout. The antigens were in this study attached more to the surface area of the PLGA particles than incapsulated. Further investigations will show whether these particles can encapsulate and release antigens in an efficient fashion, without the chronic peritonitis as seen when using oil-adjuvant vaccines

In this study we have chosen to use LPS as a positive control, since it is a known stimulant in fish.

1.3. Objectives

To develop vaccines with higher Th1 stimulating ability we chose to use PLGA carriers as they have been shown to increase vaccine efficacy against intracellular pathogens in mammalian studies [56, 59]. Before analysing their Th1 stimulating activities, we were interested in whether the PLGA could induce immune responses *per se*. Subsequently, expression of acute phase cytokines either alone or in conjunction with a known stimulant, namely bacterial lipopolysaccharides (LPS) was analyzed. In addition, a prerequisite for a high vaccine efficacy is a high-level MHC presentation of peptides by APC that occurs after uptake and degradation of e.g. a pathogen or antigen loaded particles. Hence uptake studies would suggest whether the particles were phagocytised or not. This would create a basic knowledge platform for further exploitation of this concept.

Thus, the aims of this study were to:

1. Investigate the level of IL-1β, IL-6, IL-8 and TNF-α1 transcripts at early time-points post-injection of PLGA alone or together with LPS.
2. Locate fluorescence labelled PLGA in tissues after intravenous (i.v.) injection.
3. Microscopically study the uptake/association of PLGA in/to peritoneal phagocytic cells following ip injection of fluorescence labelled PLGA particles.

2. Materials and methods

2.1. Materials

2.1.1. Animals

A total number of 50 Atlantic salmon (*Salmo salar)* with an average weight of 80 g was used. The fish were supplied by Aqua Gen Norway AS, Hemne AS and kept at Havbruksstasjonen I Tromsø AS, Kårvika. The light regime was 12 h light/day, and the freshwater temperature was kept at 4^0C. The fish were healthy, had not been vaccinated and were fed with 3 mm Skretting Nutra parr LB (Skretting AS, Norway) (appendix 1). Procedures involving animals and their care were conducted in conformity with institutional guidelines complying with national and international law and regulations.

2.1.2. Lipopolysaccharide (LPS)

LPS was extracted from *Aeromonas salmonicida ssp salmonicida* by use of the phenol-water extraction method [87] at the institute of marine biotechnology at the Norwegian College of fishery science, UiTØ. Protein contamination was measured to be 0.25 %.

See appendix 2 and 3 for chemicals and solution tables

2.2. Methods

2.2.1. Making of PLGA particles

The making of PLGA particles was done according to Davda and Labhasetwar (2002) with modifications. A 2% PVA solution was prepared in cold dH_2O, saturated with 25 uL chloroform and centrifuged at 1000 rpm (Labofuge 400R) for 5 min and then filtered through a 0.22 µm filter to remove any undissolved PVA.

PLGA particles with 6-coumarin were prepared using the water-in-oil-in-water (W/O/W) emulsification solvent evaporation method according to Davda and Labhasetwar (2002) with some modifications. This method has been used extensively for encapsulation of antigens. Here we made empty particles labelled with 6-coumarin, a fluorescent labelling, to ensure tracing of the particles. Primary solution water-in-oil (W/O) was prepared; 300 mg PLGA was dissolved in 6 ml chloroform. 100 µl of a stock solution (0.5 mg/ml (6-coumarin/chloroform)) was added to 6 ml chloroform in advance, equivalent to 15.8µg/ml.

Nine hundred µl of 10% PVA was added in two portions to the PLGA solution by vortexing for 1 min after each addition. The suspension was then cooled on ice for 5 min.
Emulsification to obtain a primary water-in-oil emulsion was performed by sonication at 35% (Sonics Vibra cell) for 2 min1.5 ml of aqueous solution containing 10% w/v PVA was added dropwise to the organic phase, while homogenizing for 2 minutes at 24000 rpm (Ultra-Turrax).The primary W/O phase was added in two portions to 40 ml 2% PVA solution with intermittent vortexing to obtain the multiple W/O/W emulsion. The emulsion was placed on an ice bath for 5 min and then sonicated at 30% for 4 min (Sonics Vibra cell).
The W/O/W emulsion was left stirring overnight on a magnetic stir plate to allow evaporation of the chloroform and formation of the particles. The suspension was then transferred to a centrifuge tube and centrifuged at 9000 rpm (9800 x g) for 10 min at 4^0C with a j.26 XP centrifuge.The particles were resuspended in distilled water and sonicated for 5 sec at 20% on an ice-bath to disperse any aggregates. Washing and centrifugation was repeated twice. These washing-steps should remove PVA-residues.
The PLGA particles were resuspended in 7 ml of trehalose after the last centrifugation and sonicated at 20% for 10 sec. Then they were transferred to a Nunc-centrifuge tube and centrifuged at 500 rpm (48 x g) for 3 min at 4^OC to remove any large aggregates. The supernatant was collected and frozen at -70^OC for 45 min and subsequently lyophilized for 2 days (Hetolab FD3). The PLGA particles were stored at 4^OC.

2.2.2. Characterization of PLGA particles

2.2.2.1. Size measurement

Distribution of size was conducted in aqueous dispersion using a dynamic laser defractometer (Nicomp). First the machine was calibrated using a DURAN bottle containing particle-free milli-Q water. The milli-Q water was filtrated through a mesh to ensure no unwanted particles are faulty taken into the calculations. All equipment used was also washed in particle-free milli-Q water. The PLGAs were diluted in a DURAN bottle and counted.

2.2.2.2. Surface characterization

A JSM-6300 scanning electron microscopy was used to determine the surface topography of the PLGAs. This work was carried out by Børge Fredriksen, PhD student, IMAB.

2.2.2.3. Fluorescent labelling

The PLGAs were resolved in 5% trehalose therafter transferred to an object glass for visual study to detect the fluorescence.

Fluorescence labelling and morphology were inspected using Leica DM600B UV-microscope and Leica application suite software (Leica Microsystems GmbH Wetzlar, Germany) against a control of non-labelled PLGA particles. The particles were inspected with the magnifier set to 40X/0, 75.

2.2.3. Distribution of PLGA particles in tissue after i.v. injection.

2.2.3.1. I.v. injection of PLGA particles into fish (pilot)

Five fish were i.v. injected in the caudal vein with 100 µl of $1x10^8$ PLGA particles/fish. Four fish were injected each time and 1 fish was kept as a control without any injections. Samples were taken after 3 h from liver, spleen, head kidney, gills and heart. This i.v. injection was repeated.

2.2.3.2. Sampling for histology.

Samples of head-kidney, liver, peritoneum, and spleen for histology were transferred to 20 ml plastic vials containing 10% formalin and kept there for 48 h. Thereafter the samples were cut into smaller pieces and put in Tamro processing embedding cassettes w/lid and soaked in 70% EtOH. Two different histology processing methods were used; 1: using xylene, and 2: a non-xylene method using isopropanol.

1. The samples were dehydrated by the help of a Shandon citadel 1000 machine. The citadel 1000 consisted of 12 wells with the following content:

1: Empty

2: Empty

3: 96% EtOH (2h)*

4: 96% EtOH (2h)

5: 100% EtOH (2h)

6: 100% EtOH (2h)

7: 100% EtOH:Xylene (1:1) (1h)

8: Xylene (1h)

9: Xylene (1h)

10:Xylene (1h)

11: Xylene: paraffin wax (1:1) (1h)

12: Paraffin wax (3h minimum)

*The period of time the samples were left in each well.

The cassettes were collected the next day. It is important that the samples are collected before the machine terminates, before the paraffin-oil congeals. Following this the tissue samples were covered with paraffin using a Leica EG 115 OH and left to cool down on an Axel Johnson CP-4 cooling plate.

The samples were cut into 5 µm sections with a Leica RM2235 and the section was transferred into a water-bath, 40^0C. The sections were collected and put on to object-glasses, dried and put in a heating cupboard at 60^0C for 30 min to loose the paraffin. The object glasses were then transferred into a xylene-bath for 5 minutes. A cover glass was mounted in a drop of histokit.

The visual analysis was done using a Leica UV-microscope. The amplifier was set to 40x/0.75 and pH 2.

2. Processing histological cuts using isopropanol.

The samples were dehydrated manually:

1. Formalin (30 min)
2. Formalin (30 min)
3. Water (30 min)
4. Isopropanol (1 h 30 min)
5. Isopropanol (2 h)
6. Isopropanol (2h)
7. Paraffin (1h)
8. Paraffin (1h)
9. Paraffin (1h 30 min)
10. Paraffin (2h)

After the dehydration-protocol, the same procedure as the previous section (histological cuts using xylene 2.2.3.2.) was followed.

2.2.4. Gene expression profiling after ip injection

2.2.4.1. Injection

The experiment was divided in 4 groups, in each case the fish were injected ip with 200 µl of respectively;

1: 0.9 % NaCl

2: PLGA nano particles (376 nm) 10^8 particles/fish

3: LPS extracted from *A. salmonicida ssp salmonicidae* (1mg/kg)

4: PLGA nano particles (10^8 particles/fish) and LPS (1 mg/kg)

The fish were sedated using benzokain (1 mg/l from a 5% stock solution) before the injection.

2.2.4.2. Sampling

Samples from the fish were collected after 2, 4, 7, 14 and 30 days. Two fish pr group at each time-point were sacrificed. The individual samples from each group were pooled before RNA isolation. Cell samples were obtained from the peritoneum and head kidney. Tissue samples from liver, peritoneum and spleen were collected, put on RNAlater and kept at -20^0C

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Collection of intra peritoneal (ip) cells

The method for collection of ip cells has been developed by Kolaczkowska (2001). At the selected time-points the peritoneal cavity was injected with 5 ml L-15 transportmedium (TM). The peritoneal region was massaged before it was cut open and the liquid was collected using a bunt 1 ml pipette-tip and transferred to a 10 ml centrifuge tube and transported back to the lab on ice. The bunt tip would prevent penetrating the organs of the peritoneal cavity.

The liquid was centrifuged for 10 minutes at 300 x g and the cell pellet was collected and resuspended in 1 ml L-15 w/0.1% FCS. This suspension was further transferred to a Nunc 24-well tray. For visual studies 100 µl of each well was transferred to wells covered with a sterile circular cover glass and 900µl L-15 w/0.1% FCS.

All wells were incubated for 1 hour at 12^OC.

The cells for visual analysis were afterwards washed with 1xPBS 3 times, and then fixation was concluded with the addition of 200 µl 10% formalin. The last incubation lasted for ½ hour at 12^OC. Again the cells were washed with PBS and the cells were collected and mounted on an object-glass using Aquamont, before further visual examination. The visual analysis was done by Leica DM600B UV-microscope and Leica application suite software.

The amplifier was set to 40x/0.75 pH 2.

The ip-cells for expression analysis were not washed but immediately after incubation added 500 µl TRIzol reagent. The suspension was transferred to new eppendorf tubes and kept at -80^{0}C until further RNA isolation.

Collection of head kidney macrophages

The fish was killed by a blow to the head and blood was depleted by using vacutainers, before opening the peritoneal cavity and removing a part of the head kidney with sterile dissection equipment.

The head kidney was cut out and transferred into a 50 ml Nunc tube containing transport medium.

In a sterile cabinet the head kidney was cut into 3-4 pieces and crushed before it was strained through a 100 µm cell strainer using 1-3 ml of TM. Further the solution was transferred to a 37 %/51 % Percoll-gradient. This gradient will separate the head kidney macrophages from the rest of the head kidney cells.

The gradient was centrifuged at 400 x g for 40 minutes at 4^{o}C using a Multifuge 1 S-R centrifuge. The cell-layer between the two layers was collected with a sterile Pasteur-pipette and washed twice with 10 ml L-15 medium. The washing procedure was done by 10 minutes centrifugation at 300 x g. The pellet was resuspended in L-15 incubation medium (IM).

The cell number was estimated to 1x 10^6 cells/ml using a Bürker chamber (Assistant) and a Nikon TmS magnifier. The cells were later transferred to a 24-well tray (Nunc) with a total of 1x10^6 cells/well. After incubation for 2 h, the cells from each group (2 fish pr group) were pooled together and added 500 µl TRIzol and frozen at -80^{O}C.

Isolation of total RNA

RNA isolation was performed according to Chomczsynski (1987), with modifications. The tissue was cut into a small piece (~30 mg), one from each individual in the group at each sampling (pooling), and transferred to a 11 ml Greiner tube containing 1 ml TRIzol. This was homogenized using an Ultra-turrax T-25 Basic and the suspension transferred to eppendorf tubes. The homogeniser was washed with chloroform→ 70% EtOH→ milliQ-water →DEPC water between each sample.

The samples were further centrifuged at 12000 x g for 10 min at 4^{O}C.

After this, both cell samples and tissue samples followed the same procedure. Two hundred µl chloroform were added pr ml TRIzol, vortexed for 15 sec at RT and incubated in RT for 5 min. The samples were then again centrifuged at 12000 x g for 15 min and 4^{O}C. The RNA containing water-phase was transferred to a new Eppendorf tube and added half the chloroform and TRIzol amount used in the preceding section. And the procedure was repeated.

The water-phase was transferred to a new Eppendorf tube and added 500 µl isopropanol to bleed off the RNA. The samples were vortexed briefly and incubated in RT for 10 minut, before centrifugation at 12000 x g for 10 min at 4^{O}C. The isopropanol was removed. The pellet was then washed with 1 ml 70% EtOH/DEPC and centrifuged at 7500 x g for 5 min. Again the liquid was removed and the pellet was left to dry on the bench. It is important not to let the RNA dry to long, but just long enough for the EtOH to evaporate. The pellet was then dissolved in 30 µl nuclease-free water and heated for 10 minute at 55-60^{O}C. The RNA was then frozen immediately at -80^{O}C.

Quality of the RNA was checked by gel-electrophoresis. For each sample the following mixture was prepared; 2 µl of RNA (sample), 4 µl nuclease-free water and 3 µl of formaldehyde loading buffer. The 1% agarose gel containing EtBr was prepared in advance. The sample mixture was loaded on to the gel, and the power supplier (Powerpack 300) was set to 250 V and 12 min. 28S r RNA and 18S rRNA would appear as two bands. Visualization was done using a GeneGenius Bio imaging system.

The amount of RNA and further quality-check was done using a nano-drop. 1.5 µl of the sample was loaded on to the spectrophotometer and measured. $A_{260/280}$ should be in the range of 1.8-2.1.

All RNA samples were DNase-treated after the quality-check to remove any genomic remnants. A TURBO DNA-free kit from Ambion was used for this purpose. A protocol from the manufacturer was used. First 0.1 volume (E.g. 25 µl sample volume was added 2.5 µ DNAse buffer, volume of each sample varied) of DNase buffer and 0.5 µl of DNase were added to each sample. The solution was mixed, centrifuged and incubated in a heating-block at 37^{O}C for 20 min. After this, 0.1 volume of DNase inactivating reagent was added and the inactivation was done by flicking the tubes 2-3 min in RT. Then the samples were centrifuged for 3 min at 10 000 x g before the supernatant was removed and frozen at -80^{O}C.

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Reverse transcriptase and Q-Real-time Reverse transcriptase polymerase chain reaction

cDNA was synthesized using Taqman RT-reagents after a protocol from a producer in GeneAmp PCR system 2700. 24 µl of RT-mastermix and 1 µl (50 ng) of RNA sample was mixed in a 0.2 ml 8-tube thermo strip and the following termic parameters were used.

- $25^{O}C$ – 10 min
- $48^{O}C$ – 30 min
- $95^{O}C$ – 5 min

The cDNA product was kept at $-20^{O}C$

Q-real-time RT-PCR was performed using duplicates and read using an ABI prism 7000 sequence detecting system. Following cycles were used:

- 1. Stage: $50^{O}C$ 2 min, 1 cycle
- 2. Stage: $95^{O}C$ 10 min 1 cycle
- 3. Stage: $95^{O}C$ for 15 sec, $60^{O}C$ for 1 min, 40 cycles
- 4. Stage: 95 ^{O}C for 15 sec, $60^{O}C$ for 20 sec, $95^{0}C$ for 15 sec.

Every PCR reaction consisted of 25 µl PCR mix, including, cDNA, sybr green PCR mastermix, water, forward and reverse primers (5µM) for IL-1β, TNF-α1, IL-8 and IL-6 respectively.

Table 2: Information of the primers used in detection of selected genes. Primers delivered from Operon (Operon Biotechnologies GmbH, Germany)

Target m-RNA	Primer	Name/Accesion no	Sequence	Tm	Amplicon
IL-1β	AsIL-1b	AY617117	CAAGCTGCCTCAGGGTCTG	83	101 (bp)
	AsIL-1b	AY617117	CGCCACCCTTTAACCTCTCC	83	101 (bp)
TNF-α1	AsTNF-a1	AY929385	TTGCTAGACTTTCCCACTGCC	79	101 (bp)
	AsTNF-a1	AY929385	TCCATGTCGCCAGTTGTCAT	79	101 (bp)
IL-6	AsIL-6	TC61685	TGGTGGTGGAGCAAAGAGTCT	82	101 (bp)
	AsIL-6	TC61685	GGAGGAGTTTCAGAAGCCCG	82	101 (bp)
IL-8	AsIL-8	DW53092	TGGAATGATTCCCCTTCTTCA	80	104 (bp)
	AsIL-8	DW53092	GCAACAGCGGTCAGGAGATT	80	104 (bp)

To quantify the result a two-fold dilution standard curve of each of the four primers (IL-1β, IL-6, IL-8 and TNF-α1) and the standard 18S was performed. The same cDNA sample was used for all the standard-curve runs.

Efficiency calculations: E = 10^ (-1/slope)

Slope and efficiency are shown in table 3.

Table 3: Slope, r^2 and efficiency for the primers. These numbers were calculated from the standard-curves (see fig. 5)

Target Gene	Slope	r^2	% E
IL-1β	-5,5038	0,9972	1,52
TNF-α1	-3,1981	0,9907	2,05
IL-6	-4,5647	0,9652	1,65
IL-8	-3,7564	0,9934	1,84
18S	-3,3415	0,9987	1,99

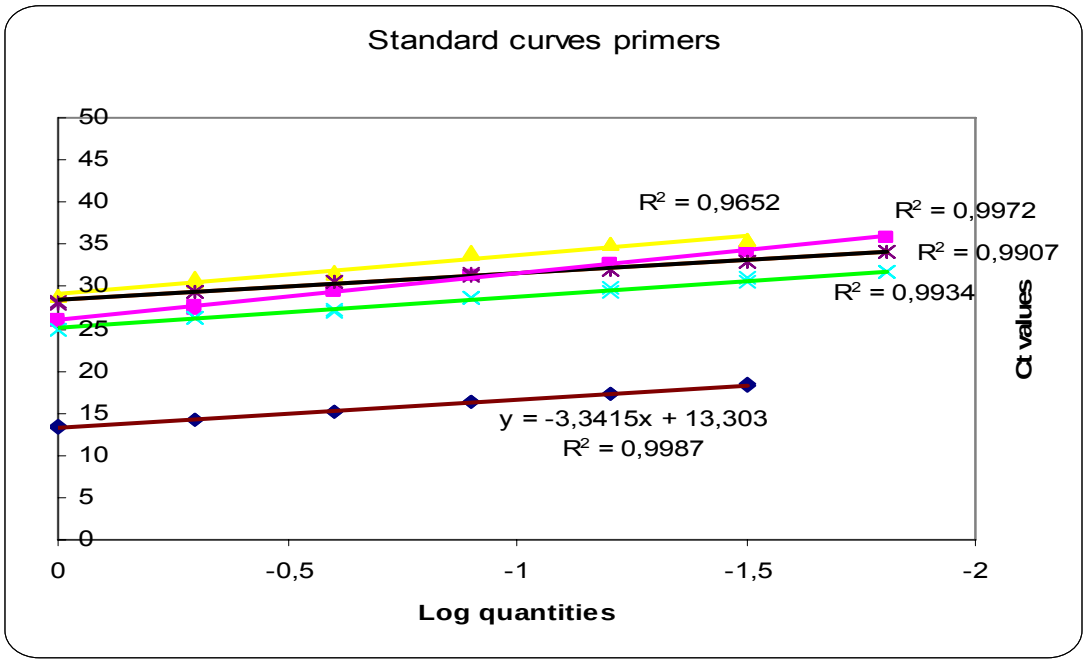

Figure 4: Standard curve for the IL-1β (pink), IL-6 (yellow), IL-8 (green), TNF-α1(black) and 18S (red) primer. The log quantities of cDNA plotted against Ct cycles gives us the calculation numbers for primer efficiency. The efficiency of the primers are listed in table 14.

The relative ratio of expression for the target gene was calculated from primer efficiency and Ct-value of the unknown sample versus the respective sample of gene of reference (18S)[72]:

$$R = \frac{E_{target}^{\Delta Ct\ target\ (calibrator\text{-}sample)}}{E_{reference}^{\Delta Ct\ target\ (calibrator\text{-}sample)}}$$

The RNA-samples were checked for any genomic contamination by running pure RNA samples through the Q-real-time-PCR machine. There was not discovered any genomic contamination.

Sybr Green

There are different approaches to monitoring DNA synthesis; we have in this master thesis used Sybr green. Sybr green is a dye that will bind to all double-stranded (ds) DNA, and gives out a bright fluorescence.

Since Sybr green binds to any double-stranded DNA it can be less specific than other methods used to monitor PCR synthesis. To ensure the specificity of the result one examines the melting curve of the products (Fig. 5). The melting point of DNA double helix depends upon its nucleotide composition, and the primers should have an specific melting-point unless there are contaminations. I.e. the samples of all products run on one primer, e.g. IL-1β, should have the same melting point at 83^{O}C. If the peaks are not similar, this can suggest contamination, mispriming or primer-dimer formations. A primer-dimer artefact is due to that the primers can sometimes anneal to themselves and create small templates for PCR amplification. Mispriming is a result of cDNA made due to annealing of the primers to complementary, or partially complementary sequences on non-target DNAs.

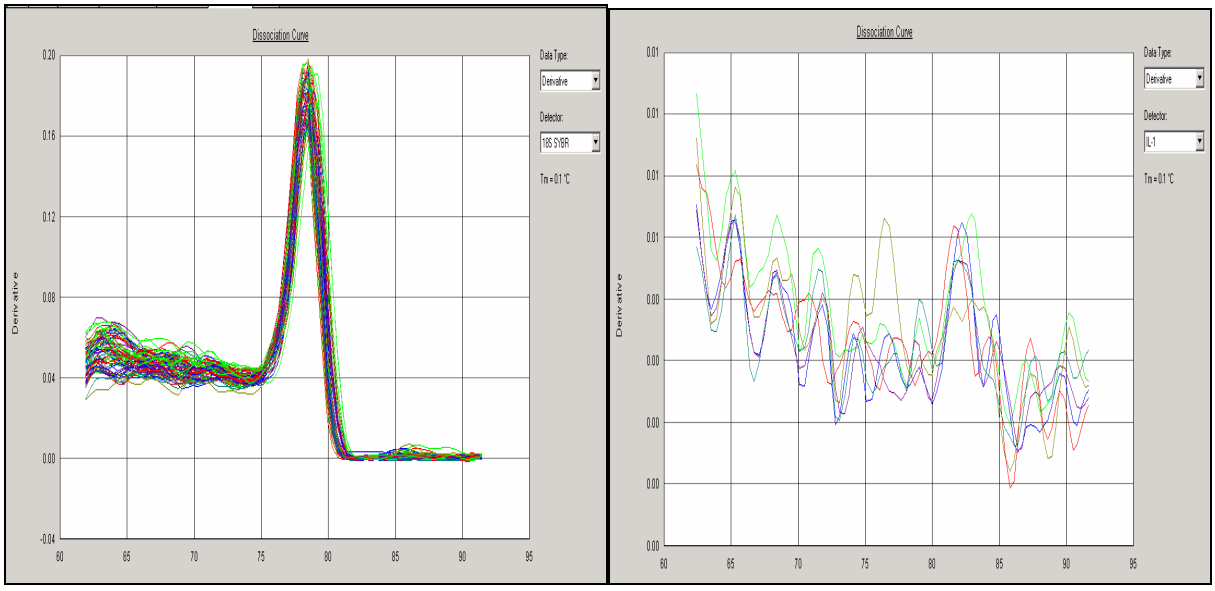

Figure 5: Disassociation/melting point (Tm) plot. The rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus the temperature on the X-axis. Left: Tm curve for 18S. The strands are showing similar melting points. Right: Faulty detection of primer-dimer artefacts are possible using sybr green, and this will give lower and fluctuated melting points due to shorter DNA strands, as shown here. Compared to the collective peak to the right.

3. Results

3.1. PLGA characterization

3.1.1. Surface characterization

To visually check the surface of PLGA particles, a Scanning Electron Microscope picture (SEM) was obtained (Fig.6 (left)). The particle size of the PLGAs, in SEM-imaging, was variable. The particles seemed to have smooth surfaces. By morphological examination the particles consisted of a mix of circular and more irregular forms. The irregular forms were probably due to particles aggregation. However the spherical forms were dominant.

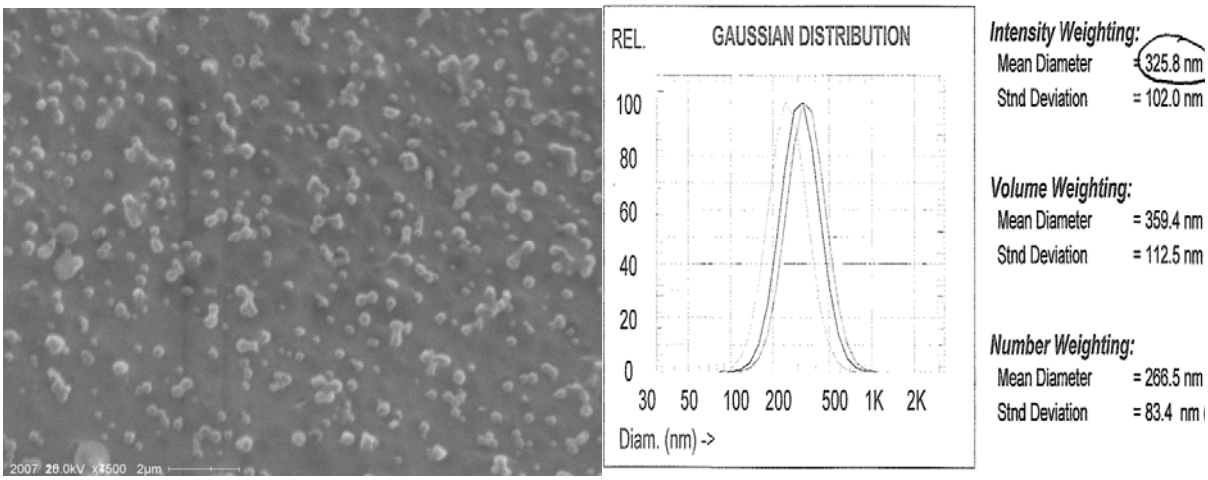

Figure 6: Left: PLGA particles Scanning Election Microscope image. Right: size distribution. PLGA particle distribution is measured by the use of Photon Correlation Spectroscopy (PCS). The mean diameter according to a Gaussian distribution is 325.8 nm.

3.1.2. Size distribution of PLGA particles

Size distribution was measured using Photon Correlation Spectroscopy (PCS). Figure 6 (right) shows a Gaussian distribution (normal distribution) of PLGA particles using PCS measurements. The bulk particles were around 300 nm, as the peak in figure 6 indicates. We see that the standard deviation was 112.5 nm.

3.2. PLGA particles associated with ip cells after ip injection

To visually study the PLGA particles in the peritoneal cavity cells, we obtained samples consisting of ip-cells at each sampling time (2, 4, 7, 14 and 30 days post-injection). Samples from all three experimental groups and the control group injected with saline were examined. PLGA particles associated to or were within ip-cells up to 14 days post-injection were observed, but no particles were seen in the other obtained experimental groups (Fig.7) Samples taken after 30 days post-injection showed no PLGA particles. Any characterizations of these ip-cells were not performed, and a quantitative measurement on the number of particles per cells was not done.

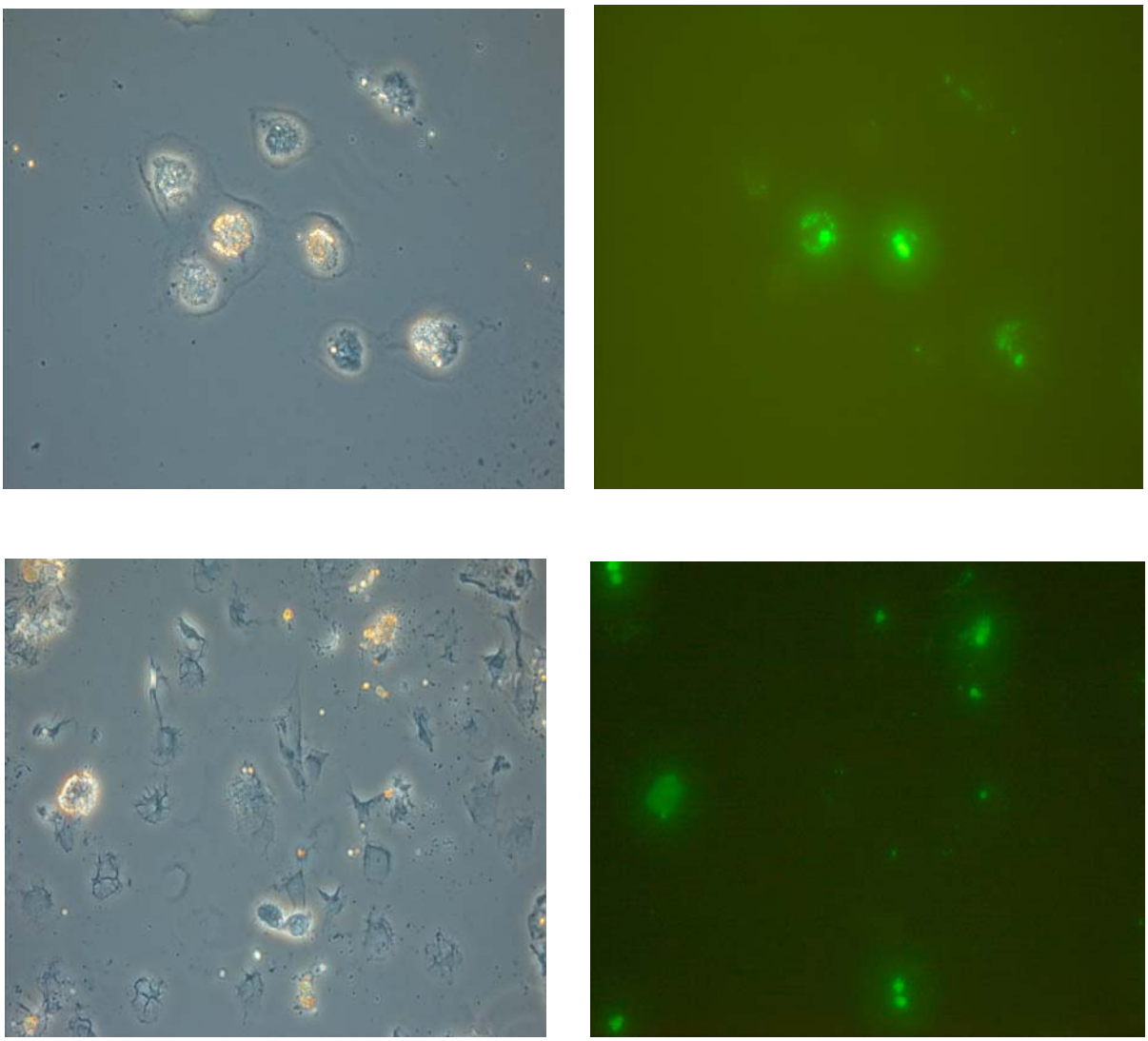

Figure 7: PLGA particles associated to or within intraperitoneal (ip)-cells after injection. Upper left: close up PH image of ip-cells sampled from group 4 (mixture of LPS and PLGA) at day 2 post-injection. Upper right: Fluorescence micrograph of the upper left cells. The 6-coumarin labelled particles appeared bright green by fluorescence microscopy. Below left: PH image of ip-cells from fish injected with PLGA/LPS at day 14 sampling. Below right: Fluorescence micrograph of the below left cells. As in the picture above we see fluorescent PLGA particles in association with ip-cells were observed.

3.3. Tissue distribution of PLGA particles after i.v. injection

PLGA particles may loose their spherical morphology and may acquire structures that may be difficult to observe by means of histological work-up of formalin fixed tissues and tissue sections. As such, we tried another dehydration procedure that replaced isopropanol with xylene during the preparation of tissue sections. No PLGA particles were observed in any tissue sections whether they were treated with xylene or isopropanol. Control sections obtained from fish injected with saline did not contain PLGA particles. However some PLGA particles were observed in blood 2 days after i.v. injection (not shown).

3.4. Cytokine expression after ip injection

The integrity of RNA isolated from intraperitoneal cells was, in some samples, poor as assessed by gel-electrophoresis. Also, the amounts of RNA isolated in a limited number of samples were low/not detectable. These RNA samples were not taken further to real-time PCR.

3.4.1. Expression of IL-1β

IL-1β is a pro-inflammatory cytokine, important in the acute-phase response. IL-1β will be secreted from macrophages at the infection site in response to different factors TNF-α1, LPS and many others. IL-1β exhibits a wide range of inflammation-inducing actions, i.e. production of other cytokines like IL-8 and IL-6, and it often co-works with TNF-α1. As such, the transcription of IL-1β was measured in samples (head kidney leucocytes, spleen and peritoneum) obtained from fish intraperitoneally injected with PLGA, LPS, and PLGA plus LPS. Control fish was injected with saline.

Expression of IL-1β was generally low in all samples obtained from either saline, PLGA, LPS and PLGA/LPS injected fish (Fig. 8). In the peritoneum all experimental groups show expression levels below the baseline level. In the liver fish injected with PLGA and a PLGA/LPS mix are showing a 2-fold increase in transcription level compared to the baseline level. The group injected with PLGA shows a slight up-regulation in expression of IL-1β, just close to 2-fold.

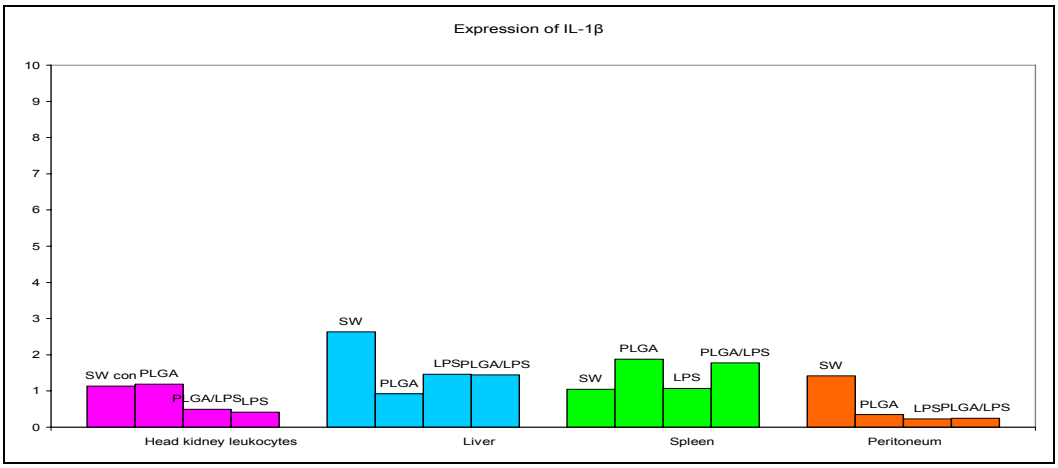

Figure 8: Relative expression of IL-1β relative to 18S in head kidney leucocytes (purple), liver (blue), spleen (green) and peritoneum (orange) after injection of saline, PLGA, LPS and a PLGA/LPS mix. The results were based on values from two parallel fish, and day 2, 4, 7, 14 and 30 results have been merged together.

Similarly, the time-course experiment for IL-1β showed that a peak expression of IL-1β in head kidney leukocytes from the saline injected controls approximately 14 days post-injection. However, there was a transient increase of IL-1β expression in samples from PLGA injected fish from day 14 to day 30 (Fig. 9)

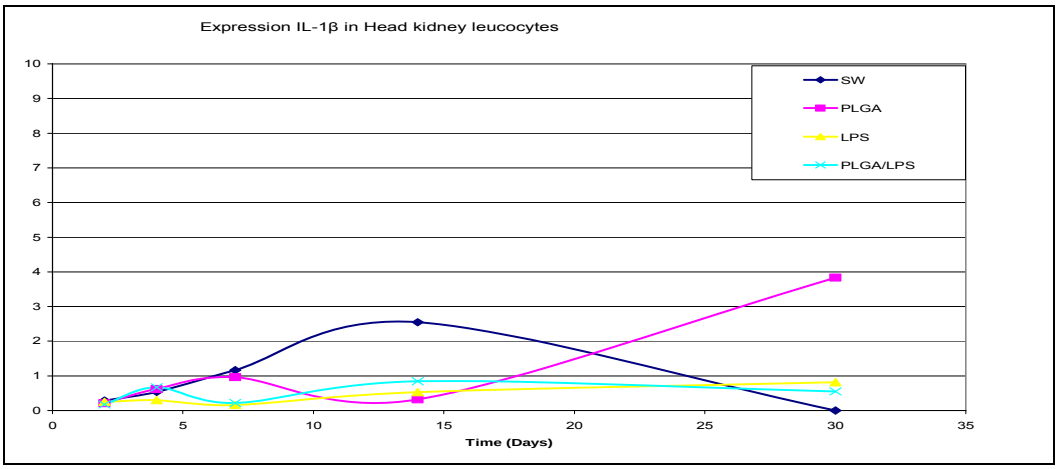

Figure 9: Time-course expression of IL-1β in head kidney leucocytes at day 2, 4, 7, 14 and 30 post injection, values were relative to expression levels of 18 S.

The time study of IL-1β expression in head kidney macrophages (Fig. 9) shows in general a low transcript level. Except from the control-group (SW) there is relatively no expression in either of the experimental groups. The highest level was from the 5th sampling, day 30 in group 2, injected with PLGAs.

As observed from head kidney cells, the expression of IL-1β in liver from saline injected fish was higher compared to the other groups and peaked at day 7 post-injection (Fig. 10). However the amount of IL-1β mRNA in liver at day 2 samples from fish injected with PLGA/LPS was higher than the corresponding saline controls (Fig. 10).

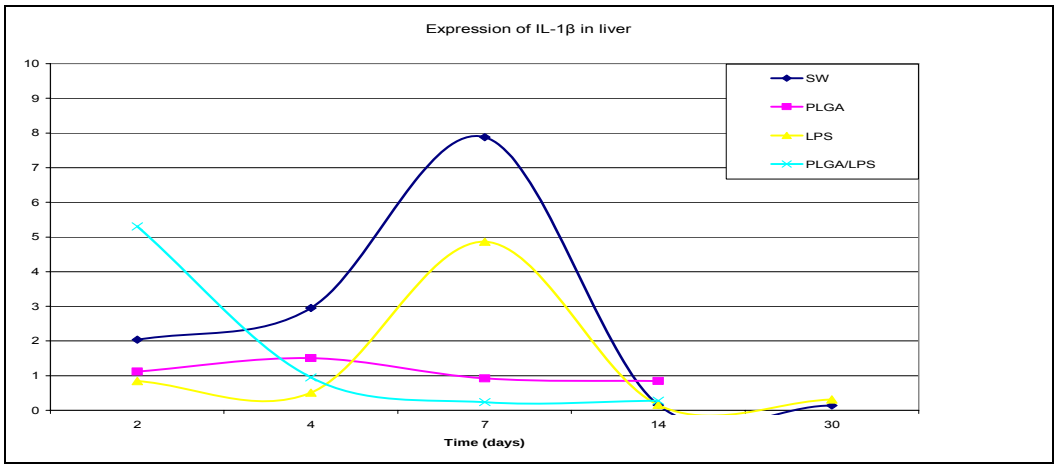

Figure 10: Time-course expression of IL-1β in liver at day 2, 4, 7, 14 and 30 post injection, values were relative to expression levels of 18 S.

In contrast to the head kidney cell and liver expression of IL-1β, the spleen expression of IL-1β in samples from PLGA and PLGA/LPS 2 days post-injection were higher than the expression levels in the controls injected with saline. The expression levels in spleen samples from the other groups were similar throughout the study (Fig. 11).

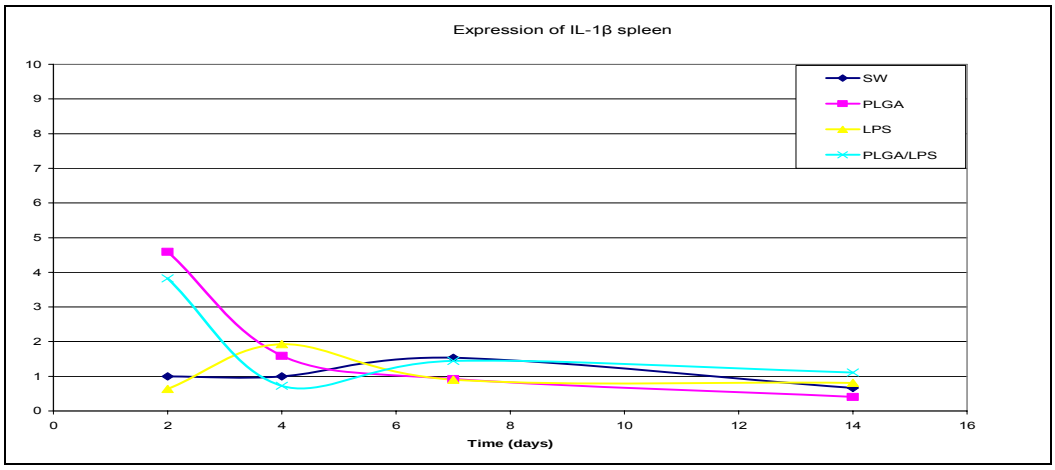

Figure 11: Time-course expression of IL-1β in spleen at day 2, 4, 7, 14 post injection, values were relative to expression levels of 18 S.

The expression of IL-1β in samples obtained from saline injected fish peaked at day 4 and 7 post-injection. The expression levels of IL-1β in samples from PLGA, LPS and PLGA/LPS treated fish were quite similar to each other at all time points investigated (Fig. 12).

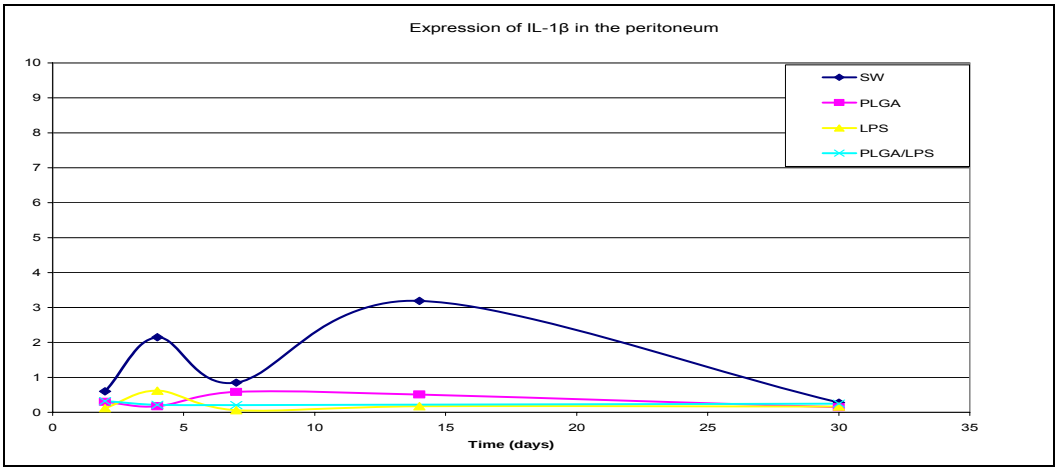

Figure 12: Time-course expression of IL-1β in the peritoneum at day 2, 4, 7, 14 and 30 post injection, values were relative to expression levels of 18 S.

3.4.2. Expression of IL-6

IL-6 is a cytokine that is central in the acute phase response. In contrast to the expression of IL-8, IL-1β and TNF-α1 in liver, IL-6 was not significantly expressed in liver of fish injected with PLGA, LPS, PLGA/LPS mix or saline. For these groups the transcript levels of IL-6 in the time-point samples (two parallel samples pooled together) were not very different from each other (Fig. 13). However the control transcript level in head kidney leucocytes was higher than the levels in samples obtained from fish injected with PLGA, PLGA/LPS mix and LPS compared to the peritoneum result (Fig. 13.)

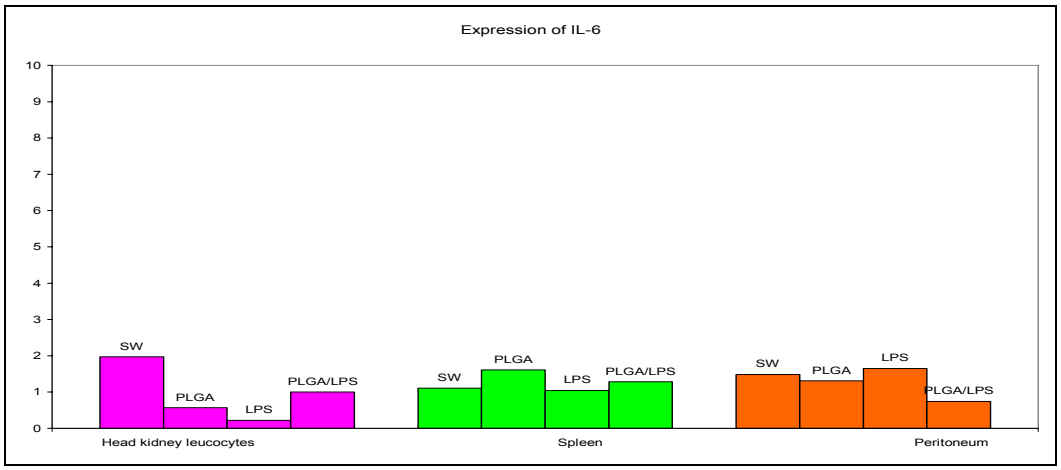

Figure 13: Expression of IL-6 relative to 18S in head kidney leucocytes (purple), spleen (green) and peritoneum (orange) after injection of saline, PLGA, LPS and a PLGA/LPS mix. The results were based on values from two parallel fish, and day 2, 4, 7, 14 and 30 results have been merged together.

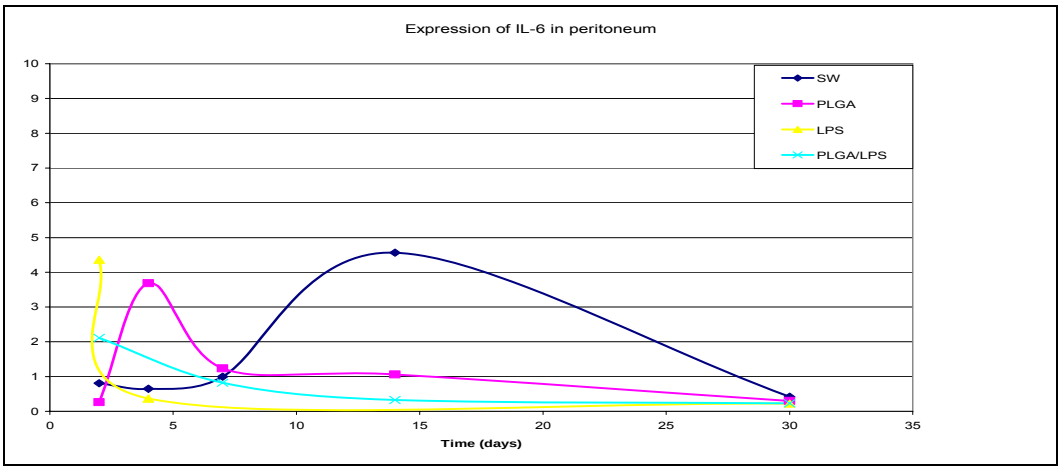

Figure 14: Time-course expression of IL-6 in the peritoneum at day 2, 4, 7, 14 and 30 post injection, values were relative to expression levels of 18 S.

As can be observed from figure 14, the expression level of IL-6 in peritoneal cells at day 2 and 4 obtained from fish injected with LPS and PLGA, respectively, were approximately four fold higher than in cells from fish injected with the PLGA/LPS mix. The relative expression of IL-6 in peritoneal cells controls increased from day 7 to day 14, whereafter reading a baseline level at day 30 post-injection.

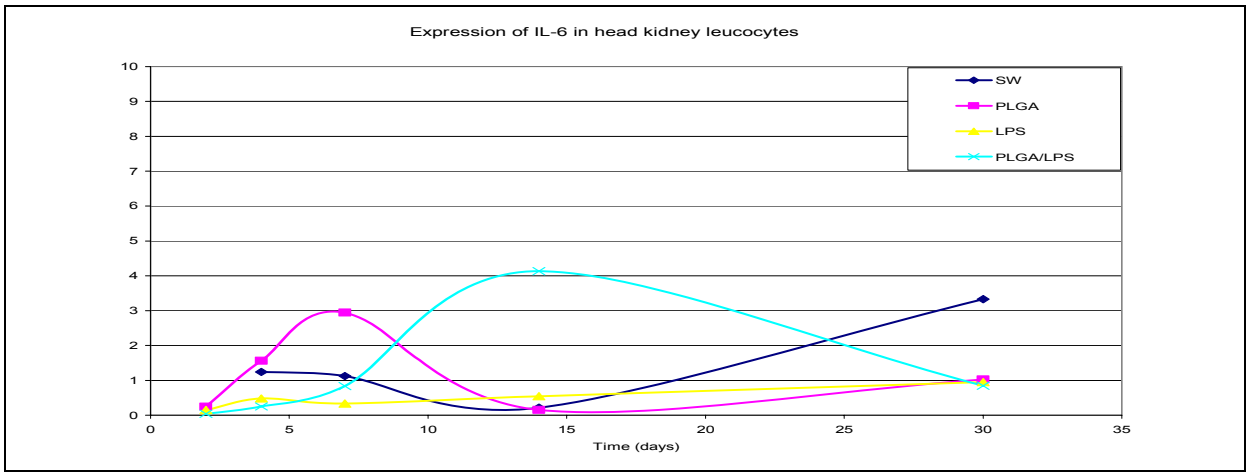

Figure 15: Time-course expression of IL-6 in head kidney leucocytes. Cells from the head kidney were sampled from fish injected with saline (SW), PLGA, LPS and a PLGA/LPS mix at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18S.

In contrast to expression levels of IL-6 in peritoneal cells, peak expression of IL-6, in head kidney leukocytes, was observed at day 7 for PLGA injected fish. Increasing levels of IL-6 transcripts were observed in cells injected with PLGA/LPS mix which peaked at day 14. Transcriptlevels in the saline injected fish increased from day 14 to day 30. The expression of IL-6 in head kidney leukocytes obtained from LPS injected fish was low throughout the study period.

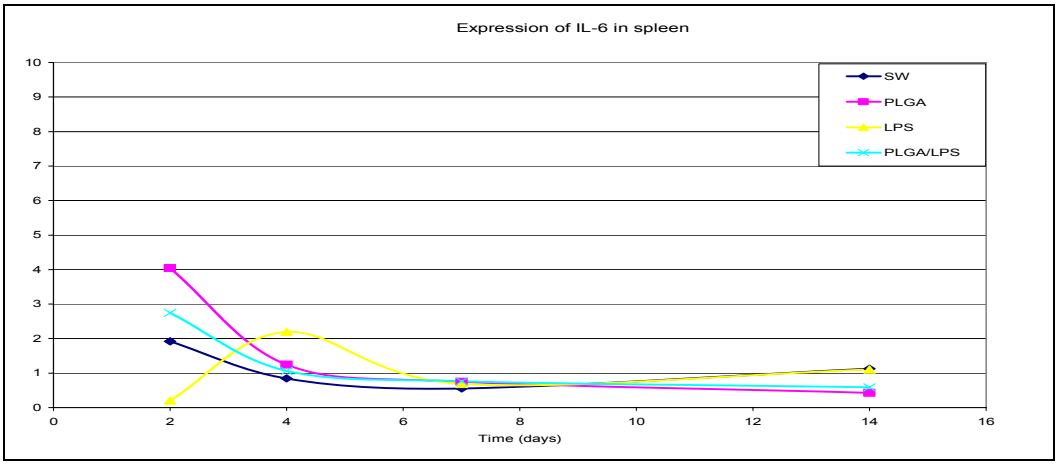

Figure 16: Time course- expression of IL-6 in spleen. Tissue from the spleen was sampled from fish injected with saline (SW), PLGA, LPS, and a PLGA/LPS mix at day 2, 4, 7, and 14 post-injection. Values were relative to expression levels of 18S.

The expression level of IL-6 in the spleen was highest in fish injected with PLGA and a PLGA/LPS mix followed by saline controls and LPS at day 2. However, LPS induced relatively high IL-6 expression in spleen obtained from fish at day 4 post-injection. Then, the expression levelled off and was similar to the transcript levels in the other groups.

3.4.3. Expression of IL-8

IL-8 is a chemokine important in the acute phase response attracting leukocytes to the inflammatory site. As such, the transcript levels of IL-8 were measured in different tissue and cell samples obtained from fish injected with the abovementioned particles with and without LPS or LPS alone. In head kidney leucocytes the expression level of IL-8 (two pooled parallels) was highest in samples from PLGA/LPS injected fish. The same was observed in spleen. Liver and peritoneal cell the transcript levels of IL-8 in fish injected with PLGA were higher than the corresponding levels in the other treatment groups. The IL-8 mRNA levels in LPS injected fish were similar or lower compared to control samples. The liver contained generally higher levels of IL-8 transcripts compared to spleen, head kidney and peritoneal cells.

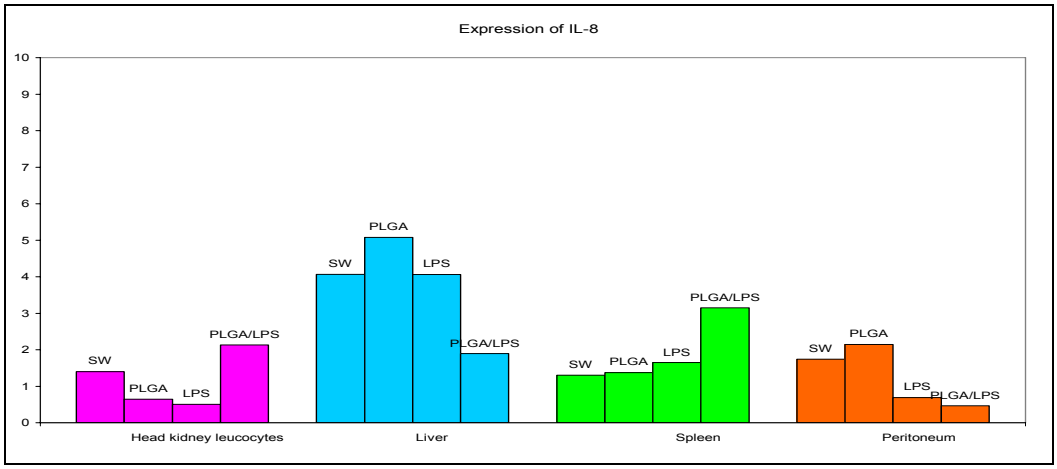

Figure 17: Expression of IL-8 relative to 18S in head kidney leucocytes (purple), liver (blue), spleen (green) and peritoneum (orange) after injection of saline, PLGA, LPS and a PLGA/LPS mix. The results are based on values from two parallel fish, and day 2, 4, 7, 14 and 30 results have been merged together.

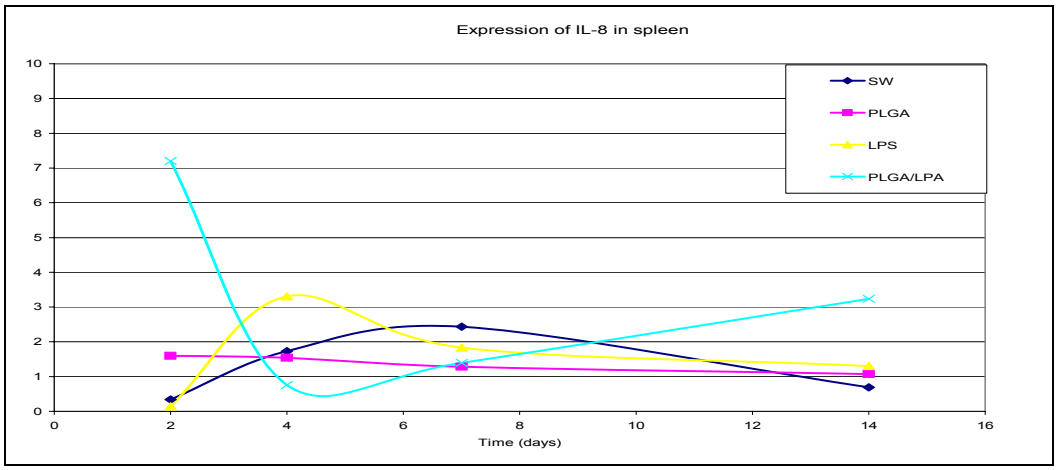

Figure 18: Time-course expression of IL-8 in spleen. Tissue from the spleen was sampled from fish injected with saline (SW), PLGA, LPS, and a PLGA/LPS mix at day 2, 4, 7, and 14 post-injection. Values were relative to expression levels of 18S.

Interestingly, the expression levels of IL-8 in the spleen of fish injected with a PLGA/LPS mixture was high at day 2 compared to the mRNA levels in the other samples (Fig.18). This peak was followed by a decrease to a base level before a minor increase to day 14. The expression levels of IL-8 in the other samples were relatively low throughout the study period, except for spleen from LPS injected fish at day 14. The expression levels of IL-8 in the other samples obtained from PLGA and saline injected fish, were relatively low throughout the study period.

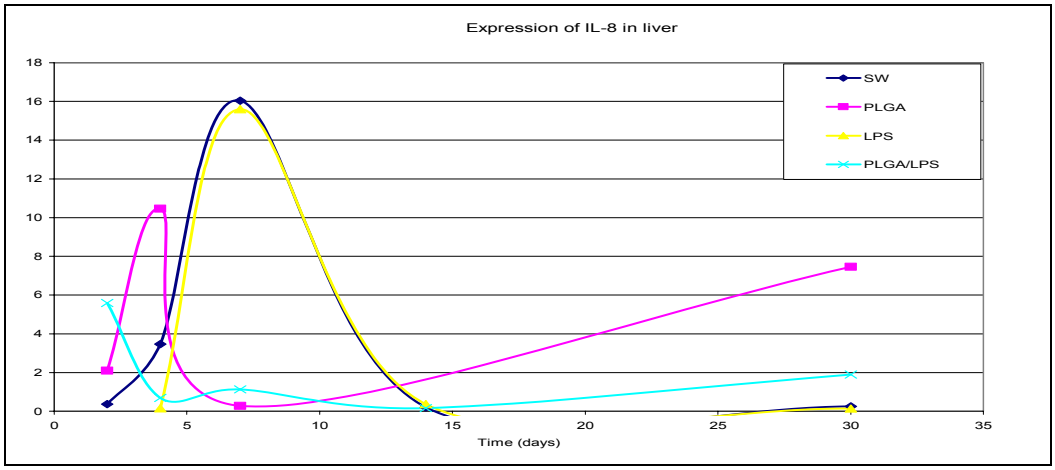

Figure 19: Time-course expression of IL-8 in liver. Tissue from the liver was sampled from fish injected with saline (SW), PLGA, LPS, and a PLGA/LPS mix at day 2, 4, 7, and 14 post-injection. Values were relative to expression levels of 18S.

The liver expression levels of IL-8 in fish injected with LPS and saline peaked at day 7 in contrast to mRNA levels in samples from PLGA and PLGA/LPS injected fish where the peak

expression was observed at day 4 and 2 post-injection, respectively. These peaks corresponded to an approximately 16-fold increase compared to a baseline value. The liver samples from PLGA injected fish showed a steady increase of IL-8 with respect to transcript levels from day 7 to day 30**.** While the transcript levels peaked at day 2 to 7 in liver samples obtained from fish injected with saline, PLGA or LPS, late peak transcription levels were found in the peritoneum (day 14 post-injection) (Fig. 20). Cells from PLGA and saline injected fish displayed increased IL-8 transcript levels compared to cells from LPS and PLGA/LPS injected fish.

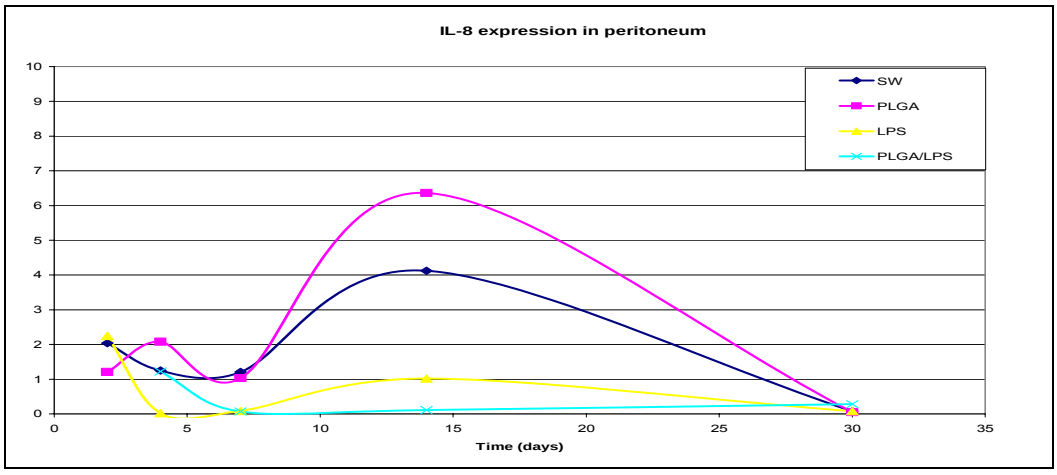

Figure 20: Time-course expression of IL-8 in peritoneum. Cells were sampled from fish injected with saline (SW), PLGA, LPS, and a PLGA/LPS mix at day 2, 4, 7, and 14 post-injection. Values were relative to expression levels of 18S.

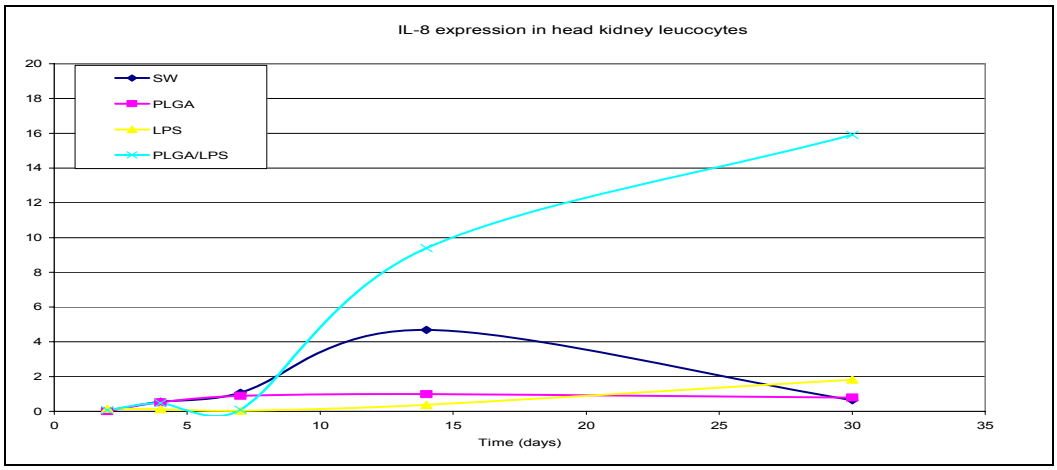

Figure 21: Time-course expression of IL-8 in head kidney leucocytes. Cells were sampled from fish injected with saline (SW), PLGA, LPS, and a PLGA/LPS mix at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18S.

The head kidney leucocyte expression of IL-8 of fish injected with a PLGA/LPS mixture did not show a peak expression of IL-8, but was increasing throughout the study period. This was in contrast to the expression profile of IL-8 in cells obtained from saline injected fish were peak expression was observed at day 14. The amounts of IL-8 mRNA found in the leucocytes from fish injected with LPS and PLGA were low during the 30-day period.

3.4.4. Expression of TNF-α1

In addition to IL-1β, IL-6 and IL-8, TNF-α1 is also considered to be an acute-phase cytokine with pro-inflammatory effects. The relative expression of TNF-α1 in the current study was generally highest in the liver and spleen. However peritoneal cells also contained significant amounts of TNF-α1 transcript levels (Fig. 22) that contrasted to the head kidney leucocytes. Overall, the liver and spleen transcripts of TNF-α1 were higher in PLGA/LPS injected fish compared to the saline injected salmon. PLGA injection induced TNF-α1 transcription in spleen and peritoneal cells however, the levels of TNF-α1 mRNA in head kidney cells obtained from saline injected fish was higher than in the corresponding levels in the other groups.

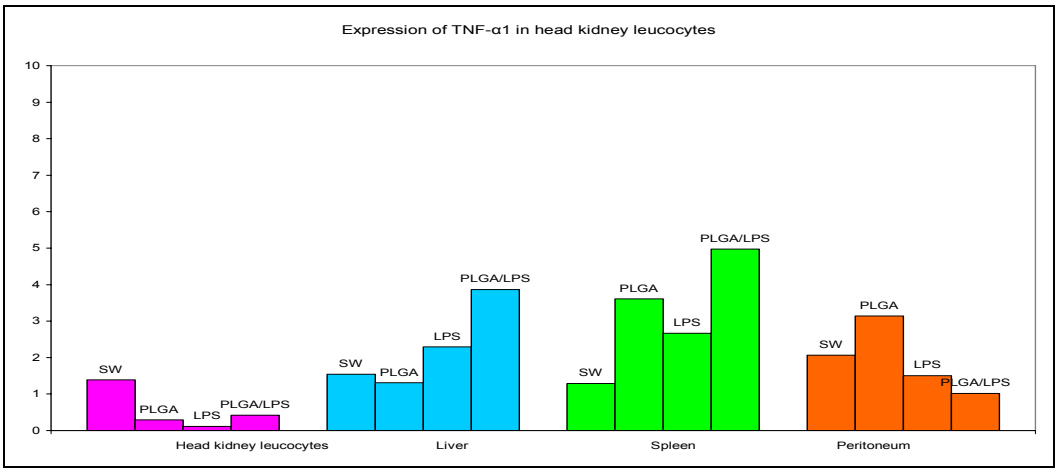

Figure 22: Expression of TNF-α1 relative to 18S in head kidney leucocytes (purple), liver (blue), spleen (green) and peritoneum (orange) after injection of saline, PLGA, LPS and a PLGA/LPS mix. The results were based on values from two parallel fish, and day 2, 4, 7, 14 and 30 results have been merged together.

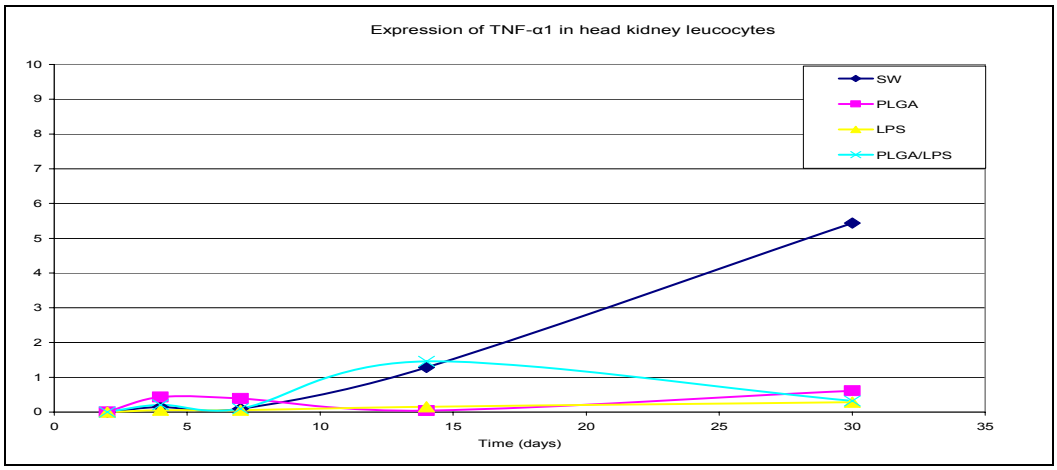

Figure 23: Time-course expression of TNF-α1 in head kidney leucocytes. Cells sampled from fish injected with saline (SW), PLGA, LPS, and a PLGA/LPS mix at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18S.

The relative expression of TNF-α1 in head kidney leukocytes were low at almost all time-points except at day 14 and 30 where cells from saline injected fish displayed relatively high levels of the gene (Fig. 23).

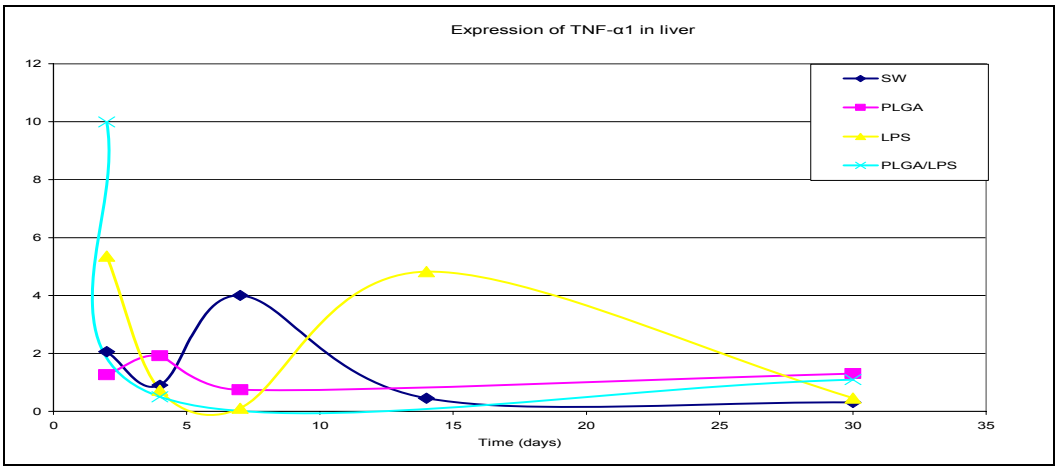

Figure 24: Time-course expression of TNF-α1 in liver sampled from fish injected with saline (SW), PLGA, LPS, and a PLGA/LPS mix at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18S.

However, the liver from PLGA/LPS and LPS injection groups (Fig. 24) showed relatively high expression of TNF-α1 mRNA 2 days post-injection where latter substance induced a peak expression also at day 14 post-injection. A peak expression was also observed in liver at day 7 for saline injected fish.

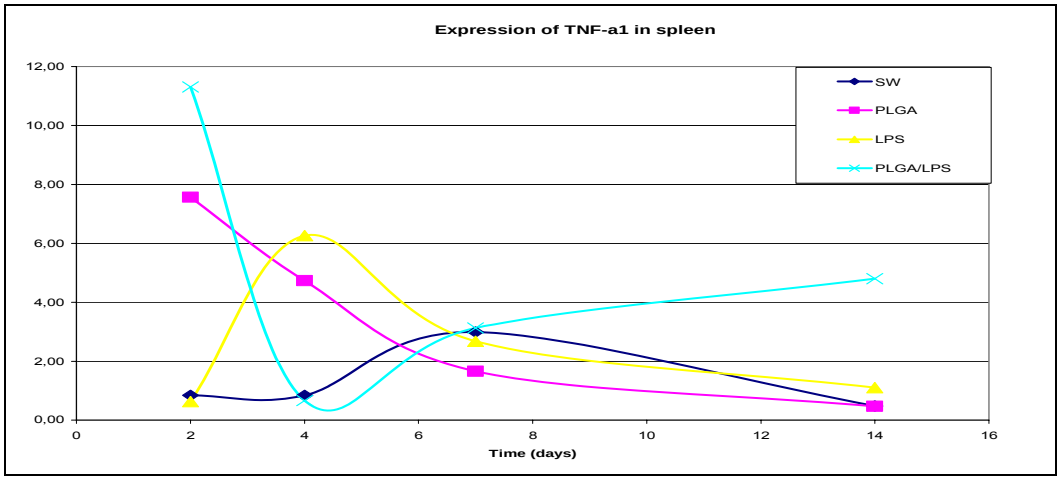

Figure 25: Time-course expression of TNF-α1 in spleen sampled from fish injected with saline (SW), PLGA, LPS, and a PLGA/LPS mix at day 2, 4, 7, and 14 post-injection. Values were relative to expression levels of 18S.

As observed with respect to liver expression of TNF-α1 in PLGA/LPS injected fish the spleen contained also relatively high amounts of TNF-α1 mRNA with a main peak at day 2 that decreased to baseline, followed by an increase throughout the study (Fig. 25). An injection of LPS induced a peak transcription of TNF-α1 at day 4, and levelled off to base-line value at day 14 as for the other fish groups. The gene expression in spleen samples from fish injected

with PLGA decreased from a near 8-fold at day 2 to base-line value at day 14. The controls contained highest level of TNF-α1 transcripts at day 7.

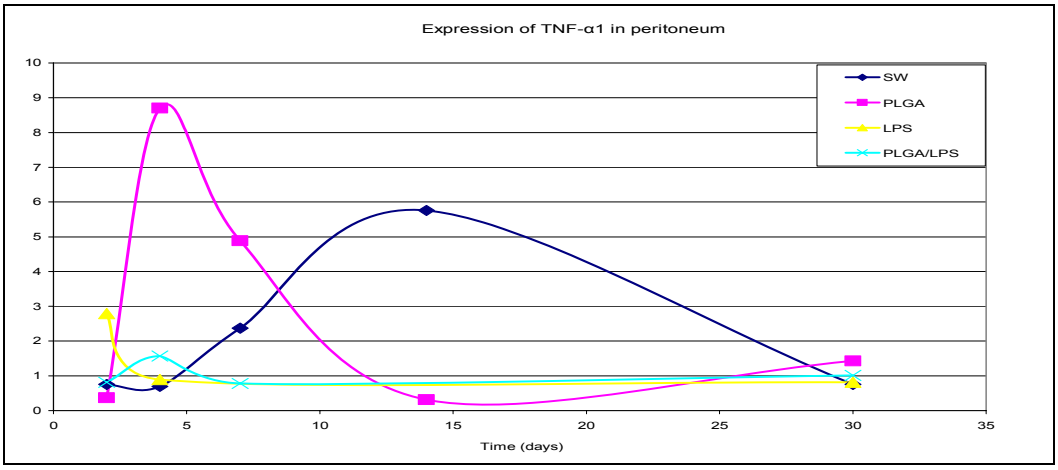

Figure 26: Time-course expression of TNF-α1 in peritoneum sampled from fish injected with saline (SW), PLGA, LPS, and a PLGA/LPS mix at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18S.

As for spleen, the transcripts level of TNF-α1 in peritoneal cells from fish injected with LPS peaked, with a 9-fold increase, early post-injection (Fig.26). This peak was followed by a decrease to day 14. The LPS induced TNF-α1 mRNA expression was relatively stable with a small peak at day 2 post-administration. Saline injection also induced TNF-α1 gene expression in peritoneal cells that peaked at day 14 post-injection. Minor changes with respect to relative expression levels were found in cells from the other treatment groups.

3.4.5. Comparison of TNF-α1, IL-1β1, IL-6 and IL-8 expression

While most of the figures 8-26 present a detailed time-course expression of one cytokine mRNA in experimental groups, I wanted to present an overall view of the time-course of all cytokines expressions in each experimental group and tissues/cell sample. As such, some details may be difficult to observe in figures 27-38.

When comparing the expression levels of the different cytokine mRNAs, within the experimental group that were injected with PLGA, in head kidney leukocytes, the overall pattern shows a general increase in expression levels from day 2 throughout the study, except for IL-8 saline control, that peaked at day 14 post-injection. Overall, the control samples showed generally higher transcript levels compared to samples from PLGA injected fish.

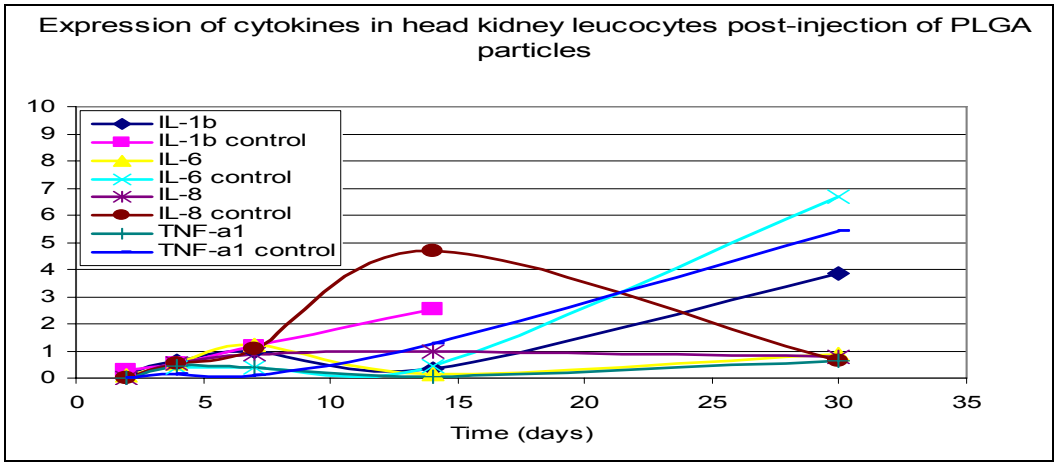

Figure 27: Time-course expression of IL-1β, TNF-α1, IL-6, IL-8 in head kidney cells sampled from fish injected with saline (control) and PLGA at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18S.

In contrast to the relative high head kidney cell expression values of saline injected control fish compared to PLGA injected fish (Fig. 27), an injection of PLGA induced spleen IL-1β TNF-α1, and IL-6 day 2 transcription levels that were relatively higher than in the respective controls (Fig. 28). At a later time-point (day 7) the general pattern was the opposite, i.e. the respective control fish showed higher spleen transcript levels of the genes than their PLGA injected counterparts. While early (< day 7) transcription levels of genes in head kidney cells was present, there was a delayed peak in transcriptions of spleen IL-1β and IL-8 (day 7) post-injection of PLGA (Fig. 28). This applied also to spleen IL-1β transcript level obtained from saline injected fish (Fig. 27).

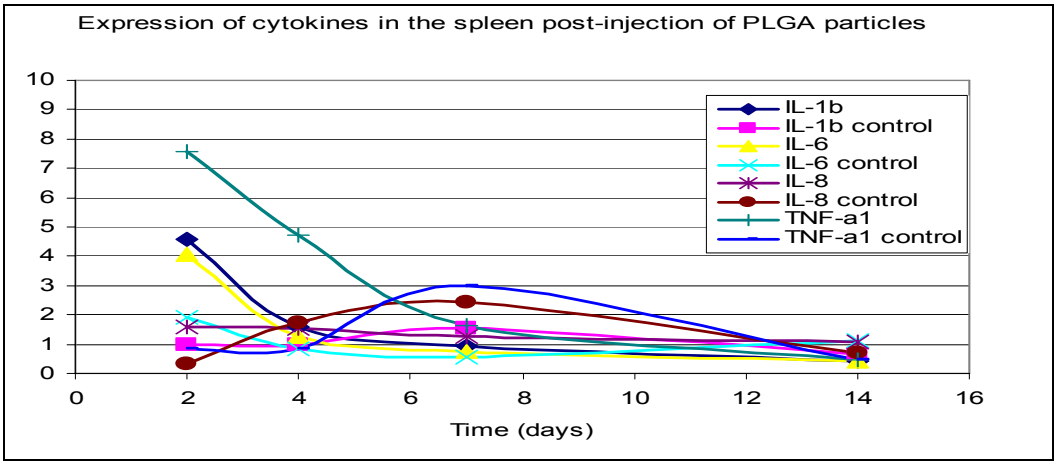

Figure 28: Time-course expression of IL-1β, TNF-α1, IL-6, IL-8 in spleen sampled from fish injected with saline (control) and PLGA at day 2, 4, 7 and 14 post-injection. Values were relative to expression levels of 18S.

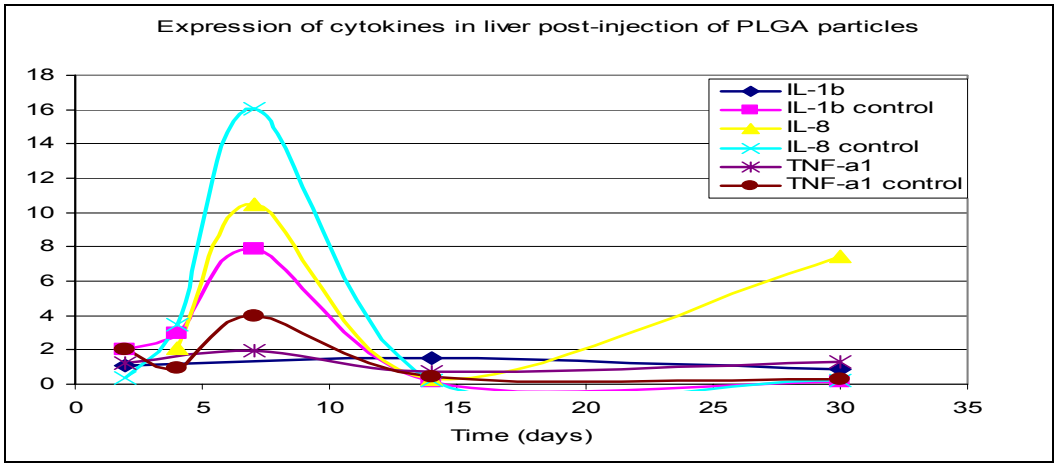

Figure 29: Time-course expression of IL-1β, TNF-α1, and IL-8 in liver sampled from fish injected with saline (control) and PLGA at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18S

In liver, the control samples obtained from fish injected with saline contained higher amounts of cytokine mRNAs at day 7 post-injection than their PLGA injected mates. In exception, the expression level of IL-8 at day 30 was higher than the IL-8 expression in liver from saline injected controls (Fig. 29)

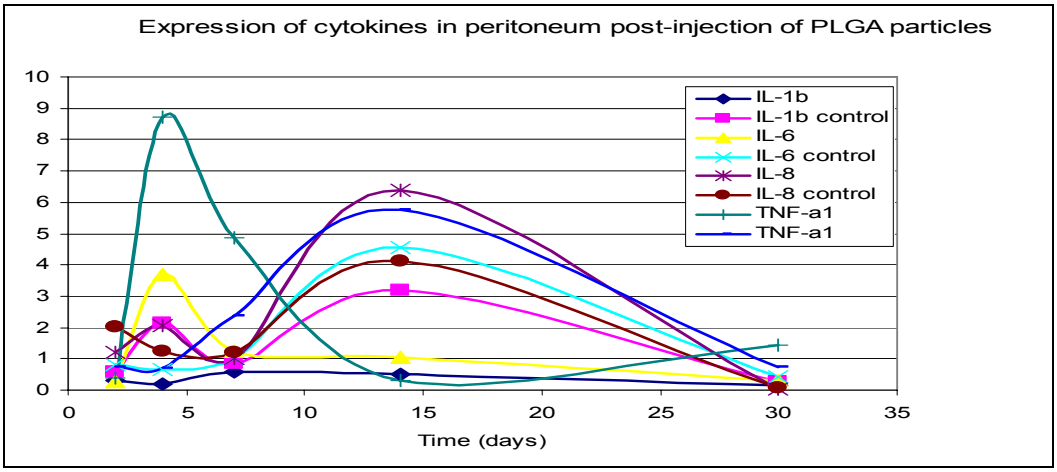

Figure 30: Time-course expression of IL-1β, TNF-α1, IL-6, IL-8 in peritoneum sampled from fish injected with saline (control) and PLGA at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18S

Peculiarly, the day 14 transcript levels of TNF-α1, IL-1β and IL-6 in peritoneal cells of fish injected with saline were higher than in cells from fish injected with PLGA. In general, the opposite was observed in the day 4 samples where the transcript level of TNF-α1, IL-6 and IL-8 in cells were higher than their respective controls (Fig. 30).

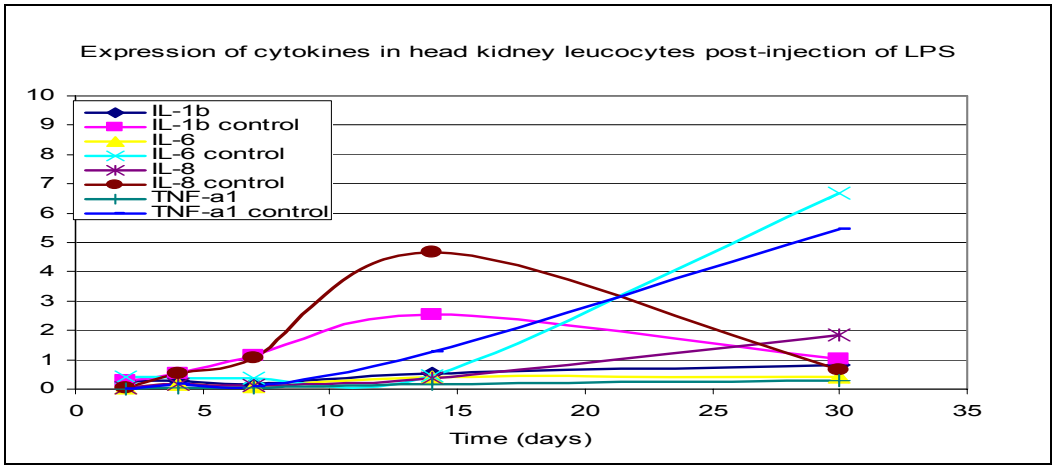

Figure 31: Time-course expression of IL-1β, IL-6, IL-8 and TNF-α1 in head kidney leucocytes sampled from fish injected with saline (control) and LPS at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18S.

Compared to spleen and liver where early peaks (day 2 to 7) with regards to transcript levels were observed, the peritoneal cell (Fig. 29) and head kidney leukocyte transcript levels of the genes under study peaked later (day 14) (Fig. 31). In the head kidney cells, the day 14 transcript level of IL-8, IL-1β and TNF-α1 were higher in saline injected fish compared to the corresponding PLGA injected fish. This was the case also in the 30 day samples for IL-6 and TNF-α1 where the controls contained higher levels of transcription levels of transcripts than their counterparts. In spleen (Fig. 32) there was a peak expression of all of the target genes at day 4 sampling, with TNF-α1 exhibiting a 6 fold-increase in expression levels compared to a baseline-level, followed by IL-8, IL-6 and IL-1β at decreasing order, and expression levels in their respective controls were lower. at day 4 sampling. These differences were not pronounced in day 7 samples, this also applied to sample obtained from fish injected with LPS 20 days previously (Fig. 32).

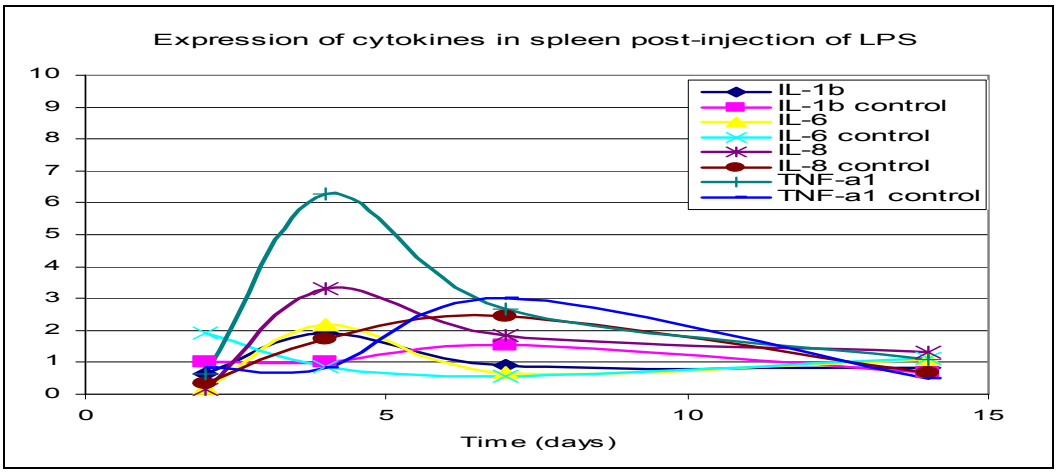

Figure 32: Time-course expression of IL-1β, IL-6, IL-8 and TNF-α1 in spleen sampled from fish injected with saline (control) and LPS at day 2, 4, 7 and 14 post-injection. Values were relative to expression levels of 18S.

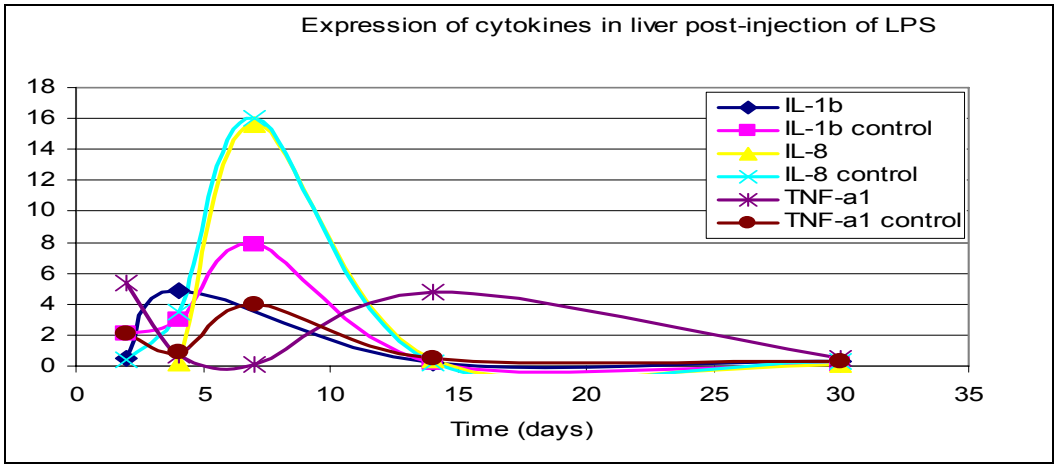

Figure 33: Time-course expression of IL-1β, IL-8 and TNF-α1 in liver sampled from fish injected with saline (control) and LPS at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18S.

Generally expression of IL-8 was higher in liver (Fig. 33) compared to spleen (Fig. 32), and also a delayed peak in comparison with the spleen was observed, with a 15-16 fold increase in expression at day 7 sampling for samples obtained from fish injected with LPS and saline, before a rapid decrease to below a baseline level at day 14 sampling. Expression of IL-8 in fish injected with saline is exhibiting the highest level, of all genes analysed, through the time-course study in liver, with a peak at day 7 sampling. Compared to the spleen, the expression levels of TNF-α1 in liver was lower, and also showed a different profile, with a peak level at day 2 sampling, before a decrease at day 4 sampling, and further an increase by

day 14.

Expression of IL-1β peaked at day 4, however it rapidly decreased to levels more or less equal to a baseline level at day 14 sampling and stayed low thereafter. Fish injected with saline also showed a peak at day 7 in expression levels of IL-1β.

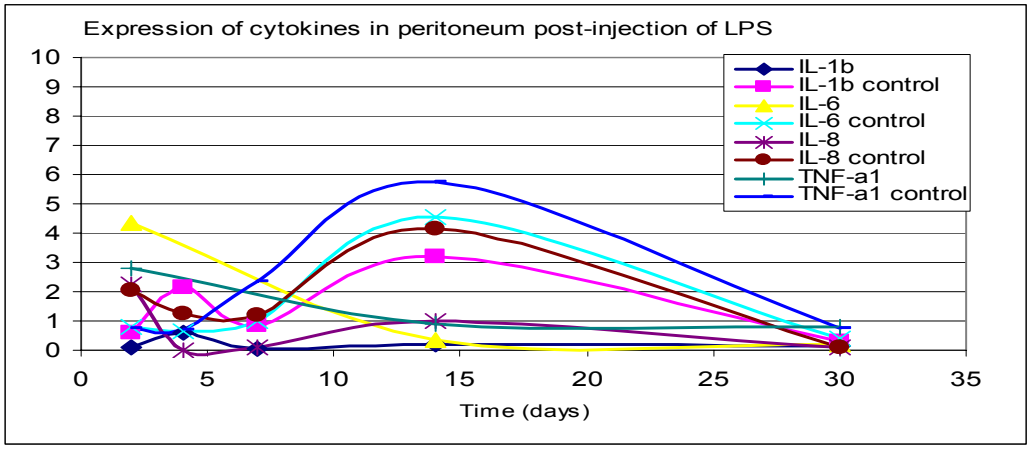

Figure 34: Time-course expression of IL-1β, IL-6, IL-8 and TNF-α1 in peritoneum sampled from fish injected with saline (control) and LPS at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18S.

Expression of cytokines in the peritoneum post-injection of LPS (Fig. 34) showed a peak in amounts of IL-6 mRNA transcripts, followed by TNF-α1, and IL-8 at day 2 sampling. IL-1β exhibited levels not significantly different to a baseline-level through the whole time-course study. However, expression of TNF-α1, IL-6, IL-8 levels in fish injected with saline peaked at day, and the peak levels exceeded the peak -levels of the experimental LPS injected fish at day 2 sampling.

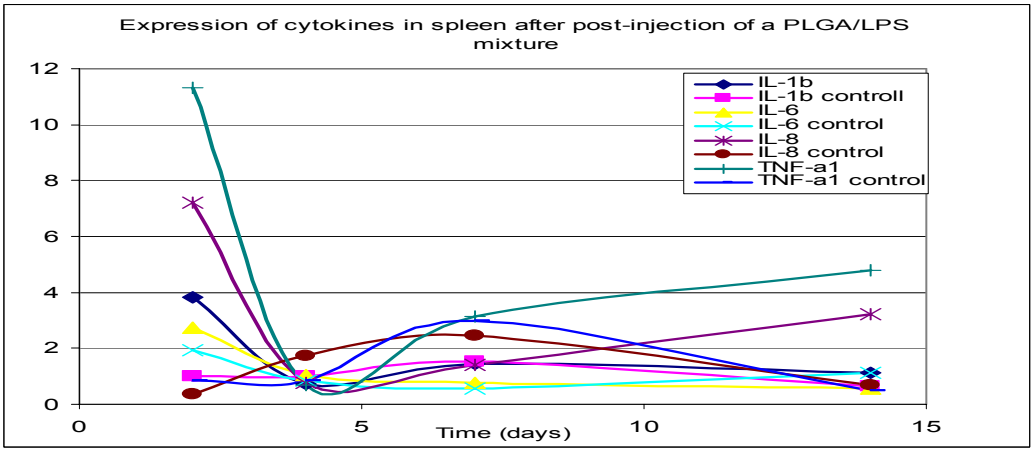

Figure 35: Time-course expression of IL-1β, IL-6, IL-8 and TNF-α1 in spleen sampled from fish injected with saline (control) and a PLGA/LPS mixture at day 2, 4, 7 and 14 post-injection. Values were relative to expression levels of 18 S.

Expression levels of TNF-α1 were high in fish injected with PLGA/LPS mixture at day 2 sampling, compared to fish injected with saline, both in spleen (Fig. 35) and liver (Fig. 36). In general, all expression levels of cytokines from the experimental group showed a peak at day 2 sampling in spleen, with TNF-α1 being the highest, and IL-8, IL-1β and IL-6 following in that order. In spleen TNF-α1 transcript levels dropped below a baseline level at day 4 sampling before a continuous increase in expression levels to latest sampling point at day 14. The target genes all decreased to a baseline level at day 7 and stays there throughout the time-course study.

In liver (Fig. 36) the same decrease in transcript levels in the PLGA/LPS experimental group was observed. However expression levels of IL-8, IL-1β and TNF-α1 rose to a peak by day 7 in sample from saline injected fish. The amounts of cytokine transcripts in PLGA/LPS and saline injected fish were low from day 14 to 30.

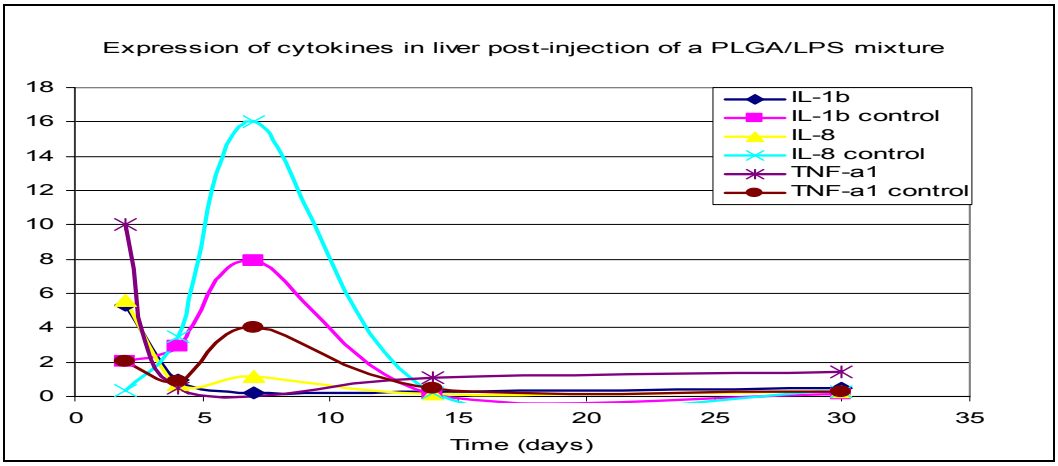

Figure 36: Time-course expression of IL-1β, IL-8 and TNF-α1 in liver sampled from fish injected with saline (control) and a PLGA/LPS mixture at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18 S.

In head kidney leucocytes two expression profiles were observed; the expression level of IL-8 increased (day 4 to 30) to a level of 16 fold higher compared to baseline level. Except from IL-8 transcript levels, there seemed to be low expression of the other cytokines studied irrespective whether they were injected with PLGA/LPS or saline. Expression levels in fish injected with saline showed increased transcript levels of IL-6 and TNF-α1 at day 30 sampling.

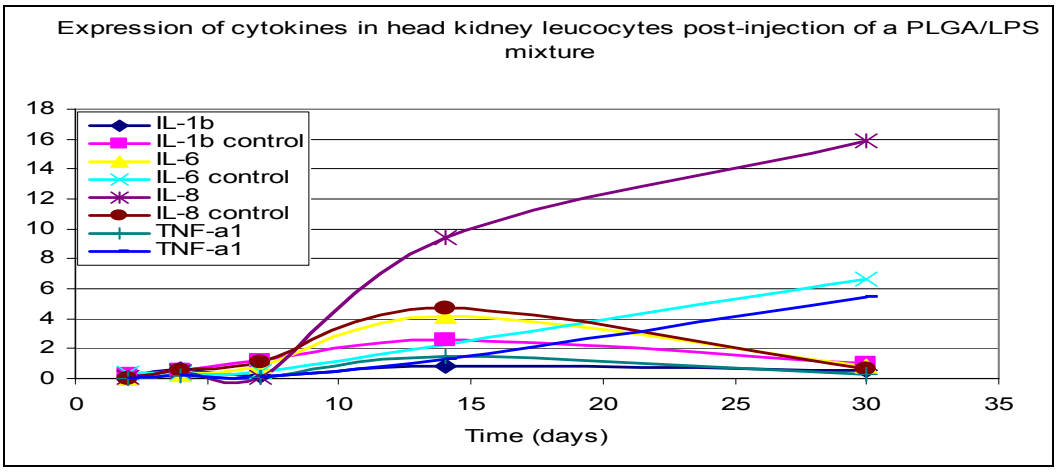

Figure 37: Time-course expression of IL-1β, IL-8, IL-6 and TNF-α1 in head kidney leucocytes sampled from fish injected with saline (control) and a PLGA/LPS mixture at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18 S.

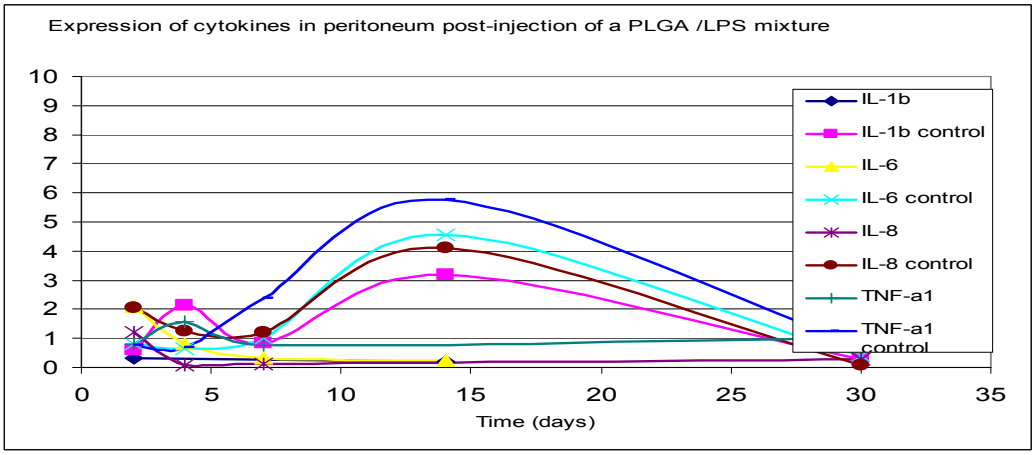

Figure 38: Time-course expression of IL-1β, IL-8, IL-6 and TNF-α1 in peritoneum sampled from fish injected with saline (control) and a PLGA/LPS mixture at day 2, 4, 7, 14 and 30 post-injection. Values were relative to expression levels of 18 S.

As seen for most cytokines studied in head kidney leucocytes (Fig. 37), there was a peak in expression level in peritoneum (Fig. 38) of IL-1β, IL-6, IL-8 and TNF-α1 at day 14 sampling from fish injected with saline. Expression levels of IL-6 at day 2 sampling showed a 2-fold increase, and compared to 18S expression throughout the time-course study expression levels were at baseline levels. Transcript levels of IL-8 and TNF-α1 were just above a baseline level at day 2 and 4 post-injection respectively, however, all levels declined to under baseline level by day 7, this included the expression levels of IL-1β and IL-6 as well.

4. Discussion

In fish there has previously been an attempt to administer antigen-containing PLGA particles orally (by intubation) [47], and the study concluded that PLGA particles were worth further investigating as an antigen delivery vehicle as the particles seemed to ensure that a larger amount of intact antigen (protein in this case) reached into the system, after passing through the stomach. Beside this, no reports are available using PLGA as vaccine carriers for use in aquaculture.

The overall aim of this thesis was to investigate the expression of pro-inflammatory cytokines, important in the early stages of inflammation, elicited by PLGA particles in LPS solution and/or alone. Repeatedly, PLGA particles as antigen carrier has been evaluated and high potencies in activating the adaptive immune response in mammals [29, 84]. However, to my knowledge there are no studies that have addressed the inflammatory response of PLGA particles *per se* by means of gene transcript analysis of acute phase products. This raised the following question: Do PLGA particles elicit immune responses on their own without antigens adsorbed onto or encapsulated inside? And does an ip injection of LPS together with PLGA induce a response higher than injections of PLGA or LPS alone.

Mammalian dendrittic cells, the main APC, showed an adjuvant-like maturation with expression of MHC class II and expression of costimulatory molecules on DC`s stimulated with PLGA particles [89]. The exact way PLGA is stimulating DC maturation in mammals is not known, like the LPS pathway to stimulate macrophage activation through the TLR4 is [34]. The level of maturation in DC`s was not at the same level as by LPS alone, but scontain macrophages (APC), whether they can respond to PLGA and LPS as shown for mouse DC [89] remains unknown.

4.1. PLGA in association with ip-cells

A prerequisite for using PLGA particles for intracellular delivery of antigens, their actual uptake by fish phagocytic cells (macrophages), the main APC in fish [20, 63]. PLGA have shown to be phagocytosed by a number of mammalian cells [65] by various endocytic routes, like phagocytosis, pinocytosis or by receptor-mediated endocytosis, and the process seems to be saturable [68, 83]. The results presented herein provide strong indications that PLGA

particles injected intraperitoneally have been taken up by phagocytic cells, the particles were seen associated or were inside the cells up to 14 days post-injection. Because the present findings are based on a limited number of fish, further experiments have to be performed to assess whether particles are also present within cells beyond 14 days. Nevertheless, the results achieved may indicate that PLGA particles offer novel delivery systems in fish by intraperitoneal administration, for a prolonged antigen release. There are reasons to believe that the cells associated with PLGA particles after 2 days are mainly neutrophils since studies have shown a great influx of neutrophils 24-48 h post-injection of bacteria in rainbow trout, about 50 times the macrophage population [2, 23]. See introduction for the cell distribution in a normal state. However the cell type responsible for uptake of PLGA has to be characterized in future studies. After injection of bacteria, a resting population of peritoneal cells was re-established within 14 days. Neutrophils have been shown to have phagocytic ability, ingesting invading microbes and then undergo cell apoptosis[1, 2]. However no conclusions are made in this study, other than, PLGA are attached to and are within ip-cells. As such, the phagocytic process itself may induce physiological changes such as modulation of acute phase cytokine transcript levels.

4.2. Cytokine m-RNA transcripts

Looking at the transcription level of pro-inflammatory cytokines, would provide us a more insight to which extent PLGA particles induce inflammation. Since no other studies of PLGA and proinflammatory responses in fish have been published, there were few previous studies to rely on. However, it is a fact that TNF-α1 and IL-1β, IL-6 and IL-8 are among the first cytokines produced in fish and mammals [5, 79], thus these cytokines are trustworthy markers in pro-inflammatory studies on fish. Biological studies of IL-1β have shown that transcript levels can be elevated by *aroA*$^{-}$ *Aeromonas salmonicida* LPS stimulation of leucocytes in rainbow trout, and inhibited by cortisol (stress-hormone) and low temperatures *in vitro* [90]. In fish IL-6, IL-8 and TNF-α expression is known to also be induced by exposure to LPS *in vitro* [32, 35, 46]. Taking these results together with observed cytokine profiles observed in mammalian species, IL-1β, TNF-α, IL-6 and IL-8 are all acute phase and are applicable to use as acute phase response markers.

4.2.1. Tools for real time-PCR

The primer efficiency was poor for three of four primers IL-1β, IL-6 and IL-8. One reason for

a low efficiency could be due to a hairpin structure in the annealing position of the primer reduces the amount of cDNA available for the polymerase. Poor quality RNA obtained from some of the samples in the current experiment were not brought to the real-time PCR. Since all primers, including those for 18S were added to the same cDNA preparation a poor quality cDNA may affect all the genes in study. Bad primer efficiency could also be due to the binding of other structures that are similar to the target genes (personal communication, Marie Løvoll). There are identified two IL-1β genes in rainbow trout [73], and perhaps can this be the case also in Atlantic salmon.

However, in this study we used a relative calculation-method [72] where transcript levels are relative to a standard (18S), thus the results can still be used to assess difference in gene-expression among the selected genes and in between tissues. There were indications of that injection of PLGA particles per se elicited low levels of all cytokines in head kidney leucocytes and liver. In spleen however, TNF-α1, IL-1β and IL-6 expression levels were elevated at early samplings (day 2), and also in the peritoneum (TNF-α1 is elevated at day 4 and IL-8 at day 14). IL-8 is known to be produced in response to IL-1 and TNF [15], therefore a link between the production of IL-1β, TNF-α1 and chemokine production could be suggested in this case.

4.2.2. mRNA transcript levels post ip injection

Results from LPS injected fish showed no detectable levels of IL-6 in liver in any experimental groups points (PLGA, LPS and a mixture of PLGA/LPS). Correspondingly, a study on rainbow trout [35] showed no presence of IL-6 mRNA in liver from untreated fish. Highest level of IL-6 in rainbow trout was in the ovary. Thus the number of particles or the amount of LPS injected in this study may not have been sufficient to induce an IL-6 expression in liver.

IL-6 holds many functions, and recent studies revealed that in acute inflammation, IL-6 decrease neutrophils migration, as described earlier, but increased monocyte/macrophage recruitment, leading to a resolution of inflammation and initiation of an immune response [40]. If we look at the expression level in peritoneum after LPS injection, IL-6 was elevated at day 4 sampling, which could be an indication of a inflammation that may induce leucocyte migration. However, the gene for IL-6 was first characterized in Japanese pufferfish *Fugu rubripes*, in 2005 [9], and in rainbow trout in 2007 [35], and whether its function as ia in

mammalian is not clear yet.

High levels of TNF-α1 were seen at day 4 in spleen and at day 2 in liver. This may indicate an early first stage elevation that induce high levels of IL-8 in liver first at day 7. As earlier mentioned, that production of IL-8 is initiated i.e. by TNF-α1, and any delayed expression of IL-8 may be due to this.

Results from fish injected with a mixture of PLGA/LPS showed elevated levels of cytokine transcripts at an early stage in spleen and liver, where TNF-α1 transcript levels were highest in both tissues, followed by IL-8 and IL-1β, IL-6 only elevated in the spleen. The expression seemed to be limited to early phases, except from TNF-α1 that in spleen was elevated again at day 14, followed by a small elevation of IL-8. TNF-α genes have been characterized and studied expression of in rainbow trout and Japanese flounder [32, 45]. In trout the TNF-α seems to be divided in two genes, as known to be the case in other molecules of salmonids. TNF-α seem to be constitutively expressed in gills and head kidney of trout as well as leucocytes in Japanese flounder. In vitro studies show up-regulation by stimulation by LPS, paramethoxyamphetamine (PMA), and trout rIL-1β receptor. Maximal expression 3-4 h post-stimulation [45]. The temperature of fish surroundings in this experiment was around $4^{O}C$, and comparison to time-line from *in vitro* studies can not be made. The temperature is low, and generally the peaks of cytokine mRNA transcript levels are around 1-4 days. One day is also included even though no samplings was made prior to day 2, however the peak in expression in especially the spleen seem to be descending at day 2 samplings to day 4, indicating a possible peak at time-points prior to 48 h. From these data there might be implications of an acute phase response in salmonids ending at day 2-4.

4.2.3. Experiment set-up and materials

The spleen and liver, which are technically easy to sample, have in overall showed more reliable results that the current real-time Hence the results from peritoneum and head kidney leucocytes were not easy to explain at all time, and in some cases, no expression levels above 18S were observed. In addition, the molecular features displayed by different LPS may result in different transcription profiles. As such *in vivo* expression studies of IL-1β and IL-8 in haddock done by Corripio- Miyar, 2006, gave a good presentation of the importance of LPS-source. The choice of LPS affects the cytokine expression after challenge. IL-1β mRNA transcripts after challenge with different *Escherichia coli* LPS, showed that expression in head

kidney, spleen, liver and gills were LPS strain dependent, and that some LPS types, did not elicit any expression. The same study showed more or less constitutive expression of IL-8 after stimulation of most of the LPS types. It was suggested that LPS variants showed different lipid A structures and therefore also induced different inflammatory responses [21]. To ensure comparable results from one experiment to the other, the source of stimulants should be kept equal in each experimental set-up.

More sampling time-points could have been added to the present study, especially the first 4 days to more closely evaluate fluctuations in mRNA transcript levels. A clear definition of when the acute phase response in fish is over aided by cytokine quantification could be interesting to evaluate correlation between mRNA transcripts and protein levels. Unfortunately, no antibodies against the studied cytokines are commercially available making cytokine quantification impossible.

The relatively high amounts of cytokine expression in saline injected fish could be due to individual differences of fish. The samples from tissues and cells were pooled from parallel fish, this may partly mask any high or low responders – or *visa versa*. By running real-time PCR on individual samples together with addition of more parallel fish may have improved present result. It is a known fact that fish do show large individual differences in immune response, thus difference in gene expression levels. The fish in this study were just days prior to injection moved from a large tank to a smaller one. The change of environment could induce stress that again may elicit up or down regulation of proinflammatory cytokines. In addition the handling involved prior to/after injection is a known stress factor for fish. The observed high transcripts levels of certain cytokines in certain samples after saline injection may be due to such a physiological process.

RT-PCR detects mRNA transcript levels, but gives no information at protein level [30]. As such an apparently early increase of e.g. TNF-α1 transcript level may not directly be comparable to physiological effects displayed by TNF-α1 itself as a mature protein. Studies of IL-1β mRNA transcription in monocytes versus macrophages after LPS stimulation, revealed that the expression level in macrophages was lower (3-folds) than in monocytes, however total protein production was higher in macrophages, suggesting a more efficient translation [85]. Studies on correlation of protein and cytokine gene expression profiles in human subjects after endotoxin challenge, revealed variable correlation between IL-1β, IL-6, IL-8 and TNF mRNA transcripts and actual detectable protein levels [76]. Cytokine activity is also

regulated on different levels; on gene-levels (transcription), on m-RNA level (translation), and on active protein level where stability of protein, presence of receptors and competing ligands decides the activity of the cytokine.

4.3. Distribution of PLGA particles

"Positive" results from a tissue-distribution study would have given clues on which organs, tissues and cells that are accumulating the PLGA particles. The whereabouts of the PLGA particles is information central to understand their impact on fish immune responses. Why the usual processing method for histology did not succeed is not certain. An earlier attempt to look at distribution of PLGA particles in salmon (not published) also failed. This was why this new study using isopropanol instead of xylene, was driven through a more or less like a pilot-study. Xylene is a powerful solvent that may disrupt the PLGA spheres, and we investigated the effects of xylene on PLGA in two ways, first by just placing particles in pure xylene which provided particles with lesser fluorescence (leakage), and then a processing method replacing xylene with isopropanol. However still after processing without xylene, no fluorescent particles could be detected another explanation is that the fluorescence label may be release from the particles shortly after injection. However fluorescent particles were found in blood after i.v. injection (3 h p.i.) in samples not or processed without xylene – (blood smear analysis). This may indicate that tissue processing for histology may disrupt the fluorescence labelled particles and impact the analysis of tissue-distribution Otherwise, isotope labelling may be a reliable way of achieving quantitative results [55]. When using radioactive labelling, precautions must be made, bearing in mind the study animals and the work environment.

4.4. Future thoughts

A different method for isolating IP-cells could have been used, i.e. a method using ergosan elicited cells to yield a higher number of cells. The Ergosan method has been used for isolating ip-cells prior to transmission electron microscopy [70]. Also by Osmium-labelling the particles, as done by Panyam *et.al* [69] time-course studies can give detailed answers to where the PLGA particles end up (this is now under study) in tissues and cells.

Further studies with cell-culture could give more answers regarding the rate of uptake and if the process is saturable as in mammals. Also when antigens have been encapsulated, it will be

important to characterize release-rate of the respective antigen.

Peritonitis is a known side-effect when oil-adjuvant vaccines are used. There was no sign of accretions in the abdominal region of the fish injected with PLGA particles and LPS (results not shown). If PLGA particles are showing promising antigen-delivery qualities in the future, this is a great advantage compared to oil-adjuvanted vaccines. Still, this was a short study, and no antigens were used.

5. Conclusion

- Fluorescent PLGA particles are associated with ip-cells up to 14 days post-injection.
- Conclusion in mRNA transcripts of cytokines being that in spite fluctuating results, PLGA seem to elicit pro-inflammatory cytokine production. A mixture of PLGA/LPS gives highest expression in most cases. Some genes seem to be easier to study transcription of than other, TNF-α being the best in this study.
- To study the distribution of PLGA particles with ordinary histology-preparation does not work. Alternative methods should be evaluated.

6. Appendix

Appendix 1: Production data

OPPRINNELSE - OG PRODUKSJONSDATA FOR FISK PRODUSERT VED LANDANLEGGET, HiT.

ART: Laks gr. 1 STAMME: LR standard · GENERASJON:2006

LEVERANDØR: Aqua Gen Norway AS, Hemne AS

KAR NR.: 5
Mottaker: Stine Mari Myren
Prosjekt nr: H06/79

	DATO	TEMPERATUR	DØGNGRADER
Innlagt	**29.11.05**	**4,1**	**396,3**
Øyerogn	-	-	-
Klekking	**20.12.05**	**7,3**	**527,3**
Startfôring	**20/2-06**	**14**	**n/a (400)**

PRODUKSJONSDATA:

	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A
Lys	0/24 :0	24:0	24:0	24:0	24:0	24:0	24:0/ 6:18	6:18	6:18	6:18	6:18	6:18	6:18	6:18	6:18	
T C	8	8/14	14	14	14	14/nat	nat	nat	nat/4	4	4	4	4	4	4	
Fôr	-	-/SK	SK	SK	SK	SK	SK	SK	SK	SK	SK	SK	SK	SK	SK	

FÔR: SK = Skretting, EW = Ewos, BI = Biomar, DF = Dana Feed.
LYS: 24:0 = kontinuerlig lys, 6:18 = 6 t lys og 18 timer mørke, 0:24= kontinuerlig mørke, osv.

Vaksinasjon: Nei Dato: ….. Vaksinetype: ….
Overført antall: 20 stk. Dato: 28/3-07. Størrelse: 57 g.
Tot. antall: ?? stk.

Status ved levering/merknader:
Vinter fra den 18/7-06.

Appendix 2: Chemicals and reagent

Table 4

Name	Manufacturer
Polyvinylalchohol (PVA) 87-89 % hydrolyzed.	Sigma Aldrich
Poly(D,L-lactide-co-glycolide) 50:50 (PLGA)	Sigma Aldrich
6-coumarin	Polyscience inc.
D-(+)-Trehalose dihydrate (Trehalose)	Sigma Aldrich
Dichlorotriazinylaminofluorescein (DTAF)	Sigma Aldrich
Fluorescein-5-thiosemicarbazide	Fluka, Sigma Aldrich
MilliQ water (dH2O)	
Dimetylsulfoxid	Fluka, Chemika
Dichloromethane (DCM)	BDH, VWR international Ltd
Benzokain	Apotekprodukt AS
Sodium Chloride	Merck
Formaldehyde 37 %	Merck
Xylene	Merck
100 % ethanol	Arcuss kjemi AS
96 % ethanol	Arcuss kjemi AS
Paraffin-oil wax	Merck
L-15 medium L-glutamine and L-amino acids	Leibowitz, Gibco, Invitrogen™
Percoll	Invitrogen, Percoll™/Redigrad™, Amersham bioscience
Sodium phosphate dibasic	Merck
Immersion oil	Cargille laboratories inc, Kebolab
Waterfree sodium phosphate dibasic	Merck
Isopropanol	Arcus kjemi AS, Norway
Heparin 2500 U / ml	Leofarm AS
1 x PBS	Gibco, Invitrogen™
Diethyl pyrocarbonat	BDH, VWR international Ltd
Qiazol Lysis reagent	Quiagen
TriZol(R) reagent	Invitrogen, Amersham bioscience
Rnasefritt dH2O	
Etidium bromide	Continental lab products
Mulit ABgarose	Multi ABgene
Formaldehyde loading buffer	Ambion
Chloroform	Merck
10X taqman RT buffer	Ambion
25mM MgCl2	Ambion
deoxyBTNs mixture	Ambion
Random hexamers	Ambion
Rnase inhibitor	Ambion
TURBO Dnase (2 units/uL)	Ambion
10X TURBO Dnase Buffer	Ambion
Dnase inactivation reagent	Ambion
Rnalater(R) solution	Ambion
Multiscribe Reverse trancriptase	Ambion
Sybr (R) green PCR master mix	Applied biosystems

Appendix 3: Solutions

Solutions for isolation of macrophages

Stock solution 90% Percoll:
Stock solution:
90 ml Percoll
(10 ml 9 % NaCl) *
0,4 ml Heparin

51 % Percoll:
56 ml stock solution
44 ml L-15 w/heparin and 2 % FCS

37 % Percoll:
41 ml Stock solution
59 ml PBS w/ 20 U/ml Heparin

L-15 w /5 % FCS*
59 ml:
PBS w/ 20 U/ml Heparin

54 % Percoll:
59,4 ml Stock solution
40,6 ml L-15 w/Heparin and 2 %FCS

25 % Percoll
27,5 ml Stock solution
72,5 ml PBS w/ 20 U/ ml Heparin

L-15 w/ 0,1 % FCS**
50 ml:
50 ml L-15
500µl FCS

*** For cod and seawater salmonids to adjust osmolarity**

**** In the following referred to as Transport medium**

***** In the following referred to as Incubation medium**

Solutions in the making of f/n-PLGA

*2% PVA	Stock solution 6-coumarin
208 µl chloroform 98 ml dH_2O (cold) 2 ml PVA Centrifuge at 1000 rpm for 5 min Filter through a 0.22 µm filter to remove undissolved PVA	5 mg 6-coumarin 10 ml chloroform Equivalent to 15.8 µg/ml

***Stock solution- 10% and 20% PVA are diluted from this concentration**

Solutions for histology and sampling of organs

10 % Formalin:

100 ml 37-40 % Formaldehyde

900 ml dH_2O

4 g sodium phosphate

6,5 g water free sodium phosphate

PLGA particle- solution

LPS solution

10^8 particles/ml: PLGA particles 3, 37um NaCl 0,9%	1 mg/kg*: LPS stock (1 mg/ml)

*Fish size were of an average of 80 g

Solutions for RNA quality check
1% Agarose gel: 4 g Multi ABgarose 3,5 uL Ethidium Bromide 400 ml TAE buffer

The Agarose gel is kept at 60^0C.

EtBr is always added right before use.

Solutions for total RNA isolation	
DEPC water: 0.5 ml Diethyl pyrocarbonat (DEPC) 500 ml Destilled water	70%EtOH/DEPC: 70 ml Absolute Alcohol 30 ml DEPC water

The DEPC water must be autoclaved before use.

Solutions for reverse transcriptase and Real-time PCR	
RT Mastermix: 2.5 µl 10 x Taqman RT buffer 5.5 µl Mg_2Cl 4.0 µl deoxyNTPs mixture 1.25 µl Random hexamers 0.5 µl Rnase inhibitor 0.625 Multiscribe™ Reverse Transcriptase (50U/µl) 1.0 µl RNA sample (50 ng/ml)	PCR mastermix 12.5µl 2 x Sybr green PCR master mix 7.0 µl Rnase free water 1.5 µl Primer forward 1.5 µl Primer reverse 2.5 µl Templat

Appendix 4: Equipment

Table 5

Equipment for making of NP	
Sonica Vibra cell VC750, 3mm tapered microtip	Sonics & materials inc, USA
Nalgene centrifuge tube, 30 ml	Nalgene, USA
j-26 XP centrifuge	Avanti®, BeckmanCoulter®, USA
Labofuge 400R	Heraus instruments, Germany
Heto FD3 freezedrier	Heto labequipment, Denmark

Table 6

Equipment for PLGA characterization	
Fluorescense analysis	
DM 6000 B Microscope	Leica microsystems, Germany
CTR 5 6000 Microscope	Leica microsystems, Germany
Object glass, 50 elka, 75x26 mm no 2400	Assistent®
Apllication suite software	Leica, Germany
Measurement of PLGA particles	
Biocapby erlab vertical sterile bench	Biocapby erlab, Germany
Submicron particle sizer model 370-s	Nicomp particle sizing systems, USA
CW388 version 1.68 software	Nicomp particle sizing systems, USA
Surface characterization	
EM FC6 cryo unit	Leica, Germany
EM UC6 ultra microtom	Leica, Germany
JEOL JSM-6300 Scanning Electron microscope	JEOL Ltd, Japan

Table 7

Equipment for injection of PLGA	
1, 5 and 10 ml sterile syringe	BD Plastipak®
Microlance 25 G needles	BD Plastipak®
Scissors for labeling/ or other labeling equipment	
Timer	

Table 8

Equipment for isolation of fish macrophages/leukocytes	
Petri-dishes	
Pipette	FINNPIPETTE, Finland
Falcon cell strainer 100µm	FALCON®
96/24 wells	BD Plastipak®
Sterile stainless surgical blades 22, no 4 fitment	Lance paragon Ltd, UK
Scalpel no 4	Comac
Scissors	
Tweezers	Comac
Vacutainer precisionglide	Beckham Pickinson vacutainer systems
Vacutainer glasses 4,5 ml	Beckham Pickinson vacutainer systems
Microlance needles 25 and 23 G	BD plastipak®
TmS Nikon Magnifier	Nikon, Sweden

50 ml nunc vial	
Bürker chamber (counting-chamber), 0,100 mm depth, 0,0025 mm^2	Assistant®

Table 9

Equipment for histology and sampling of organs	
20 ml plastic vials	Zinsser analytic, Germany
Scissors	
Scalpel no 4	Comac
Sterile stainless surgical blades 22 no 4 fitment	Lance Paragon Ltd, UK
Tweezers	Comac
Vacutainer precisionglide	Beckham Pickinson vacutainer systems
Vacutainer glasses 4,5 ml	Beckham Pickinson vacutainer systems
Microlance needles 25 and 23 G	BD plastipak®
Dehydration machine Citadel 1000	Shandon, USA
Leica EG 115 OH paraffin-oil machine	Leica, Germany
Immersion Oil	Kebo Labs
Cover glass, circular and rectangular Elka	Assistant®
Object-glass, 50 Elka, 75x26 mmr no 2400	Assistant®
HI 1210 Water bath	Leica, Germany
RM2235 Cutter	Leica, Germany
Heating cupboard	Termaks, Norway
CP-4 cooling board	Axel Johnson lab systems, Denmark

Table 10

Equipment for collection of IP-cells	
Specialized pipet tips for IP-Cells[1]	FINNTIP®
5 and 10 ml sterile syringe	BD Plastipak®
Microlance needles 27 G	BD Plastipak®

Table 11

Equipment for visual analysis	
DM 6000 B Microscope	Leica, Germany
CTR 6000 microscope	Leica, Germany
Application suite software	Leica, Germany
Circular cover-glass	Knittel glaser, Germany
Rectangular cover-glass 24x24 mm rectangular	Knittel glaser, Germany
Object-glass, 50 Elka, 75x26 med mer no 2400	Assistant®

Table 12

Equipment for total RNA isolation	
Greiner tubes	
Ultra-turrax® T25 Basic	IKA®, Werke, Norway
Eppendorf	
Heating board	Termaks, Norway
Eppendorf Centrifuge 5417	Eppendorf BB lab AS, UK

Table 13

Equipment for Reverse transcriptase and Real-Time PCR	
ABI prism 7000 sequence detection system	Applied Biostystems, USA

7000 systems SDS software	Applied Biosystems, USA
Microamp™ optical 96-well reaction plate w/barcode	Applied Biosystems, USA
Microamp™ Optical Adhesive films	Applied biosystems,USA
Heating board	Termaks, Norway
Abgene 0.2 ml Thermo-strip, 8 tubes and caps	Abgene, UK
Geneamp® PCR systems 2700	Applied Biosystems, USA
Centrifuge	

Table 14

Equipment for RNA quality check	
Biorad power pack 300	Biorad, Norway
Biokey Screener 8 gel chamber	Biokeystone, US
Genius Bio imaging systems	Syngene, UK
Genesnap software	Syngene, UK
Nanodrop® ND-1000 spectrophotometer	Nanodrop technologies, US
ND-1000 software	Nanodrop technologies, US

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